Global Pattern of Gene Expression of *Xanthomonas axonopodis* pv. *glycines* Within Soybean Leaves

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To better understand the behavior of *Xanthomonas axonopodis* pv. *glycines*, the causal agent of bacterial pustule of soybean within its host, its global transcriptome within soybean leaves was compared with that in a minimal medium *in vitro*, using deep sequencing of mRNA. Of 5,062 genes predicted from a draft genome of *X. axonopodis* pv. *glycines*, 534 were up-regulated in the plant, while 289 were down-regulated. Genes encoding YapH, a cell-surface adhesin, as well as several others encoding cell-surface proteins, were down-regulated in soybean. Many genes encoding the type III secretion system and effector proteins, cell wall-degrading enzymes and phosphate transporter proteins were strongly expressed at early stages of infection. Several genes encoding RND multidrug efflux pumps were induced in planta and by isoflavonoids *in vitro* and were required for full virulence of *X. axonopodis* pv. *glycines*, as well as resistance to soybean phytoalexins. Genes encoding consumption of malonate, a compound abundant in soybean, were induced in planta and by malonate *in vitro*. Disruption of the malonate decarboxylase operon blocked growth in minimal media with malonate as the sole carbon source but did not significantly alter growth in soybean, apparently because genes for sucrose and fructose uptake were also induced in planta. Many genes involved in phosphate metabolism and uptake were induced in planta. While disruption of genes encoding high-affinity phosphate transport did not alter growth in media varying in phosphate concentration, the mutants were severely attenuated for growth in soybean. This global transcriptional profiling has provided insight into both the intercellular environment of this soybean pathogen and traits used by *X. axonopodis* pv. *glycines* to promote disease.

Xanthomonas axonopodis pv. *glycines* is a foliar pathogen causing bacterial pustule disease, one of the most serious diseases of soybean. Disease symptoms include numerous small pustules, formed by hypertrophy and hyperplasia of parenchyma cells (Groth and Braun 1986), surrounded by chlorotic halos on leaves. Like other foliar pathogens, *X. axonopodis* pv. *glycines* presumably persists as an epiphyte on the surface of healthy plants before entering the intercellular spaces (apoplast) of soybean via natural openings such as stomata or wounds. Once in the apoplast,

in which they obtain sufficient nutrients to multiply to high population sizes, they incite disease symptoms (Jones and Fett 1985).

Several virulence factors have been described in *X. axonopodis* pv. *glycines*, including the exoenzymes cellulase, protease, endo- β -1,4-mannanase, and pectate lyases as well as the secreted virulence factors indole acetic acid and cytokinin (Hokawat and Rudolph 1993; Kaewnum et al. 2006; Thowthampitak et al. 2008). Like that of many other plant pathogens, *X. axonopodis* pv. *glycines* uses a type III secretion system (T3SS) to secrete effectors such as *avrXgl* into the host cell (Athinuwat et al. 2009; Kim et al. 2003). *X. axonopodis* pv. *glycines* also employs several different global regulators to coordinate expression of target genes in a context-dependent manner. Cell density-dependent behaviors are coordinated by a diffusible signal factor-dependent quorum sensing system encoded by the *rpf* locus (Thowthampitak et al. 2008). In addition, a novel LuxR homolog, XagR, was recently shown to differentially affect expression of *pip*, *yapH*, and at least 77 other genes during the infection event, in response to feedback from plant molecules (Chatnaparat et al. 2012). While certain virulence genes of *X. axonopodis* pv. *glycines* have been previously identified, little information is available on their patterns of expression during interaction with the plant.

A variety of methods have been used recently to identify genes in several different plant-pathogenic bacteria that are up-regulated in planta. *In vivo* expression technology (IVET) was performed to identify genes of *Pseudomonas syringae* pv. *tomato*, *Erwinia chrysanthemi*, *Erwinia amylovora*, *Ralstonia solanacearum*, and *Xanthomonas campestris* pv. *campestris* that are up-regulated in their plant host during the infection process (Boch et al. 2002; Brown and Allen 2004; Osbourn et al. 1987; Yang et al. 2004; Zhao et al. 2005). Likewise, a recombinase-based *in vivo* expression technology was used to identify genes in *X. campestris* pv. *vesicatoria* upregulated during its interaction with tomato (Tamir-Ariel et al. 2007). A variant of the IVET system termed habitat-inducible rescue of survival was used to identify some plant-induced genes in *P. syringae* pv. *syringae* (Marco et al. 2005). While these methods have provided insight into environmentally induced changes in gene expression of several plant-pathogenic bacteria, most provide an incomplete inventory of gene expression, restricted to those genes that are relatively highly expressed, and can identify only those genes that show upregulation (but not downregulation). DNA microarray-based techniques have, thus, been used more recently to study global gene expression patterns of *Ralstonia solanacearum*, *P. syringae* pv. *syringae*, and *X. oryzae* pv. *oryzae* in planta (Jacobs et al. 2012; Soto-Suárez et al. 2010; Yu et al. 2013) but still suffer somewhat

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from lack of sensitivity for transcripts that are of low abundance. Global transcriptome analysis using direct mRNA sequencing (RNA-Seq) can achieve unprecedented levels of accuracy and specificity in quantifying gene expression. Moreover, RNA-Seq does not depend on genome annotation for prior probe selection and avoids the related biases introduced during hybridization of microarrays. While RNA-Seq has proven to be a powerful technique used to characterize expression of all the genes from several plant-pathogenic bacteria under in vitro conditions (Hockett et al. 2013; Liu et al. 2013), it has received little application for studies in planta because of the need to selectively isolate transcripts of the pathogen relative to that of host RNA so that sufficient cDNA sequences could be obtained to enable accurate estimates of the abundance of pathogen transcripts.

To better understand the behavior of *X. axonopodis* pv. *glycines* within soybean, its global transcriptome was assessed, using deep sequencing of mRNA. Methodologies for selective recovery of pathogen transcripts were developed that enabled accurate estimates of the abundance of even relatively rare transcripts to be assessed. The patterns of expression of a variety of genes provided insight into the apoplastic environment of soybean pathogen and the contribution of some of the plant-inducible genes of *X. axonopodis* pv. *glycines* to its virulence were demonstrated.

RESULTS

Transcripts of *X. axonopodis* pv. *glycines* 12-2 in soybean leaves.

To identify genes of *X. axonopodis* pv. *glycines* that were either up- or down-regulated during its interaction with soybean, the global transcriptome of *X. axonopodis* pv. *glycines* within soybean leaves was compared with that in a minimal medium in vitro, using RNA sequencing. *X. axonopodis* pv. *glycines* 12-2 was inoculated into soybean and was subsequently collected after 3 days, while cells grown in M9 minimal medium were harvested at mid-exponential phase (optical density at 600 nm [OD₆₀₀] = 0.6). Sequencing of three replicate cDNA samples of *X. axonopodis* pv. *glycines* recovered from soybean yielded 40.6, 60.1, and 77.6 million reads and 57.76, 60.24, and 57.19% of the sequence reads could be aligned to open reading frames present in the *X. axonopodis* pv. *glycines* draft genome. Of the 5,062 genes of *X. axonopodis* pv. *glycines*, 823 were significantly differentially expressed and by at least fourfold when compared with that when grown in M9 minimal medium. Of these, 534 were up-regulated in the plant while 289 were down-regulated (Supplementary Table S1).

To confirm the differential expression of these genes, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis was carried out on aliquots of the same RNA samples used in RNA-Seq. Ten selected genes exhibited the same relative gene expression as that found during RNA-Seq analysis, with a Spearman's rank correlation coefficient (*R*) at 0.8061 (Supplementary Fig. S1).

Annotation of genes differentially expressed in planta.

Given that a large number of *X. axonopodis* pv. *glycines* genes were up-regulated within the apoplast of soybean relative to that in a minimal medium, a thorough annotation of these genes was performed to ascertain those that could most directly be linked to the virulence of this pathogen. Several different virulence genes, including the *hrp* cluster encoding machinery for the T3SS, putative effectors delivered by the T3SS, and host cell wall-degrading enzymes (CWDE) were strongly up-regulated. Among the differentially regulated genes of *X. axonopodis* pv. *glycines*, several known as well as novel putative virulence factors related to that in other *Xanthomonas* spp. or other pathogens were up-regulated in soybean (Table 1). These included

the regulators *hrpG* and *hrpX* (XagID1272-1273), which encodes an important regulatory protein of the *hrp* cluster (XagID415-440), and genes encoding avirulence and type III effector proteins, such as an avirulence protein that is homologous to those in the AvrBs3/phA family, and the putative type III effector HolPtoQ. In addition to the genes encoding Hrp/T3SS, other pathogenicity genes linked to the type II secretion system (T2SS) were also found to be up-regulated in soybean. The genes encoding various CWDE, including polygalacturonase (PG) (XagID691, XagID2260), xylanase (XagID972), xylanase precursor (XagID4015), and xylosidase/arabinosidase (XagID2392) were expressed at a higher level in planta. Such plant CWDE are secreted by the T2SS (Ryan et al. 2011).

Several genes encoding multidrug efflux pumps were up-regulated in *X. axonopodis* pv. *glycines* within the apoplast. Efflux pumps are part of a complex system important in the processes by which toxic compounds are tolerated as well as in trafficking small molecules involved in interactions with hosts, cell homeostasis, intercellular signaling, and virulence (Burse et al. 2004; Maggiorani Valecillos et al. 2006; Martinez et al. 2009; Piddock 2006; Ravirala et al. 2007; Vargas et al. 2011). Several *X. axonopodis* pv. *glycines* genes encoding multidrug efflux pumps were strongly induced in planta. For example, three operons encoding RND (resistance-nodulation-division) multidrug efflux pumps (XagID2687-2689, XagID342-343, and XagID1501-1504) were expressed more than fourfold higher in the soybean apoplast compared with that in minimal medium. In addition, genes predicted to encode other multidrug resistance systems (XagID1460-1462 and XagID2334-2336) were both much more highly expressed in soybean than in vitro.

Several genes in *X. axonopodis* pv. *glycines* that enable it to utilize carbon resources in soybean were up-regulated in the apoplast. Such genes included those encoding a Mg⁺⁺/citrate complex transporter (XagID3278), succinate-semialdehyde dehydrogenase (XagID2330), the fructose-specific IIB component of the phosphotransferase transporter carbohydrate transport system (XagID2367), endoglucanase precursor protein (XagID3305), gluconolactonase (XagID577), 2-dehydro-3-deoxygalactonokinase (XagID1758), glucoamylase (XagID3038), and malate synthase (XagID269). Perhaps most noteworthy was the finding that genes in the operon encoding malonate decarboxylase (XagID590-596) enabling consumption of malonate, a compound particularly abundant in soybean, were strongly induced in planta.

A variety of genes involved in phosphorous metabolism were up-regulated in *X. axonopodis* pv. *glycines* in the apoplast. Several genes in *X. axonopodis* pv. *glycines* involved in phosphate transport, including those encoding the PhoR-PhoB two component system (XagID1081-1082) that regulate the phosphate-specific transport (Pst) system (*pstSCAB-phoU* operon) (XagID1577-1583) were significantly induced in soybean. In addition, the gene encoding a putative phytase precursor (XagID2378) was also up-regulated in soybean. It has been reported that *phyA*, a gene encoding phytase A of *X. oryzae* pv. *oryzae* is involved in promoting utilization of phytic acid as a phosphate source and contributes to its virulence in rice (Chatterjee et al. 2003). *X. axonopodis* pv. *glycines*, thus, apparently also perceives low phosphate conditions in the apoplast, necessitating the scavenging of phosphate from phytic acid.

Several genes involved in the putative response of *X. axonopodis* pv. *glycines* to chemical stresses were induced in the apoplast, suggesting that it experiences these conditions during the infection process. During the infection process, reactive oxygen species are often produced in plants that can cause substantial damage to all cellular components of the pathogen as a defense against pathogen attack. Peroxiredoxins and bacterioferritin are a family of antioxidant proteins ubiquitously conserved in a wide

Table 1. Differential expression of genes in *Xanthomonas axonopodis* pv. *glycines* 12-2 during growth in soybean

GeneID	Function	Ratio (plant/M9)	
		Fold change (log ₂)	P value
TypeIII secretion system (T3SS)			
Xag1272	HrpG protein	1.07	0
Xag1273	HrpX protein	3.79	0
Xag415	HpaF protein	2.63	0
Xag416	HrpF protein	4.70	0
Xag417	FIG01211675; hypothetical protein	1.15	2.49E-40
Xag418	Type III effector	0.14	0.019568
Xag419	HpaB protein	5.07	0
Xag420	HrpE protein	3.08	0
Xag421	HrpD6 protein	5.58	0
Xag422	HrpD5 protein	6.15	0
Xag423	HpaA protein	4.28	0
Xag424	Type III secretion (T3SS) inner membrane protein (YscS, homologous to flagellar export components)	4.81	0
Xag425	T3SS inner membrane protein (YscR, SpaR, HrcR, EscR, homologous to flagellar export components)	3.98	0
Xag426	HrcQ protein	5.20	0
Xag427	HpaP protein	5.63	0
Xag428	T3SS inner membrane channel protein (LcrD, HrcV, EscV, SsaV)	3.83	0
Xag429	T3SS inner membrane protein (YscU, SpaS, EscU, HrcU, SsaU, homologous to flagellar export components)	4.95	0
Xag430	HrpB1 protein	7.40	0
Xag431	HrpB2 protein	6.20	0
Xag432	T3SS bridge between inner and outermembrane lipoprotein (YscJ, HrcJ, EscJ, PscJ)	5.61	0
Xag433	HrpB4 protein	5.74	0
Xag434	T3SS inner membrane protein SctL	5.97	0
Xag435	T3SS cytoplasmic ATP synthase (EC 3.6.3.14, YscN, SpaL, MxiB, HrcN, EscN)	5.21	0
Xag436	HrpB7 protein	4.02	0
Xag437	T3SS inner membrane protein (YscT, HrcT, SpaR, EscT, EpaR1, homologous to flagellar export components)	4.32	0
Xag438	T3SS outermembrane pore forming protein (YscC, MxiD, HrcC, InvG)	4.14	0
Xag439	Hpa1 protein	6.73	0
Xag440	Hpa2 protein	3.19	0
Xag4086	Candidate type III effector HolPtoQ	2.26	0
Xag(1)_100	Candidate type III effector HolPtoQ	4.74	0
Xag301	Avirulence protein	3.35	0
Xag3053	Avirulence protein	2.25	0
Xag(1)_26	Avirulence protein	5.26	0
Xag(1)_411	Avirulence protein	1.22	0
Xag(1)_59	Avirulence protein	2.49	0
Xag(1)_862	Avirulence protein	0.37	1.00E-22
Multidrug resistance efflux pumps			
Xag1501	RND efflux system, membrane fusion protein CmeA	2.71	0
Xag1502	RND efflux system, inner membrane transporter CmeB	2.41	0
Xag1504	RND efflux system, outer membrane lipoprotein CmeC	1.66	0
Xag2361	RND efflux system, membrane fusion protein CmeA	1.44	0
Xag2362	RND efflux system, inner membrane transporter CmeB	0.41	6.21E-83
Xag2644	Acriflavin resistance protein	-0.52	1.25E-06
Xag2687	RND efflux system, outer membrane lipoprotein CmeC	5.79	0
Xag2688	RND efflux system, inner membrane transporter CmeB	6.76	0
Xag2689	RND efflux system, membrane fusion protein CmeA	8.21	0
Xag2690	Transcription regulator of multidrug efflux pump operon, TetR (AcrR) family	0.37	1.90E-24
Xag3116	RND multidrug efflux transporter; Acriflavin resistance protein	-0.99	1.98E-208
Xag3117	Membrane fusion protein of RND family multidrug efflux pump	-0.75	1.19E-117
Xag342	RND efflux system, membrane fusion protein CmeA	3.30	0
Xag343	RND efflux system, inner membrane transporter CmeB	1.82	1.32E-168
Xag3738	Acriflavin resistance protein	-1.05	5.90E-226
Xag(1)_186	Acriflavin resistance protein	-0.31	4.36E-98
Xag(1)_817	RND efflux system, membrane fusion protein CmeA	1.38	1.25E-59
Xag(1)_822	RND efflux system, inner membrane transporter CmeB	1.89	0
Xag(1)_914	RND efflux system, outer membrane lipoprotein CmeC	5.75	0
Multidrug resistance, tripartite systems			
Xag1460	Outer membrane component of tripartite multidrug resistance system	1.85	0
Xag1461	Membrane fusion component of tripartite multidrug resistance system	2.14	0
Xag1462	Inner membrane component of tripartite multidrug resistance system	0.66	7.40E-98
Xag2334	Outer membrane component of tripartite multidrug resistance system	2.85	0
Xag2335	Inner membrane component of tripartite multidrug resistance system	2.09	0
Xag2336	Membrane fusion component of tripartite multidrug resistance system	2.30	0

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Table 1. (continued from previous page)

GeneID	Function	Ratio (plant/M9)	
		Fold change (log ₂)	P value
Carbon metabolism			
Malonate decarboxylase			
Xag590	Malonate decarboxylase α subunit	3.85	0
Xag591	Malonate decarboxylase delta subunit	3.24	0
Xag592	Malonate decarboxylase β subunit	3.24	0
Xag593	Malonate decarboxylase γ subunit	3.31	0
Xag594	Phosphoribosyl-dephospho-CoA transferase (EC 2.7.7.-)	4.03	0
Xag595	Triphosphoribosyl-dephospho-CoA synthetase (EC 2.7.8.25)	3.68	0
Xag596	Malonyl CoA acyl carrier protein transacylase (EC 2.3.1.39)	2.95	0
Fructose utilization			
Xag2365	Phosphoenolpyruvate-protein phosphotransferase of the phosphotransferase transporter system (PTS) (EC 2.7.3.9)	3.57	0
Xag2366	1-phosphofructokinase (EC 2.7.1.56)	3.29	0
Xag2812	Phosphoenolpyruvate-protein phosphotransferase of the PTS (EC 2.7.3.9)	0.93	4.12E-271
Xag2367	PTS system, fructose-specific IIB component (EC 2.7.1.69)/PTS, fructose-specific IIC component (EC 2.7.1.69)	2.74	0
Xag939	Transaldolase (EC 2.2.1.2)	0.02	0.01446
Methylcitrate cycle			
Xag1144	Propionate catabolism operon regulatory protein PrpR	0.25	4.28E-06
Xag1145	Methylisocitrate lyase (EC 4.1.3.30)	3.32	0
Xag1146	2-Methylcitrate synthase (EC 2.3.3.5)	1.22	0
Xag1147	2-Methylcitrate dehydratase FeS dependent (EC 4.2.1.79)	1.42	0
D-Galacturonate and D-Glucuronate utilization			
Xag170	4-Deoxy-L-threo-5-hexosulose-uronate ketol-isomerase (EC 5.3.1.17)	-0.38	2.36E-22
Xag171	2-deoxy-D-gluconate 3-dehydrogenase (EC 1.1.1.125)	0.07	0.043192
Xag2260	Polygalacturonase (EC 3.2.1.15)	4.98	0
Xag3355	Pectate lyase precursor (EC 4.2.2.2)	-0.47	5.72E-10
Xag351	2-Keto-3-deoxygluconate permease	1.49	0
Xag4014	Uronate isomerase (EC 5.3.1.12)	1.27	0
Xag4018	Hexuronate transporter	0.46	0.000184
Xag691	Polygalacturonase (EC 3.2.1.15)	6.20	0
Xag150	2-Dehydro-3-deoxygluconate kinase (EC 2.7.1.45)	1.36	0
Xag2056	4-Hydroxy-2-oxoglutarate aldolase (EC 4.1.3.16)/2-dehydro-3-deoxyphosphogluconate aldolase (EC 4.1.2.14)	0.02	0.035184
Xag2462	α-Glucosidase (EC 3.2.1.20)	-1.18	0
Xag2407	Ketoglutarate semialdehyde dehydrogenase (EC 1.2.1.26)	2.49	0
Xag4011	Endo-1,4-β-xylanase A precursor (EC 3.2.1.8)	-0.46	3.19E-38
Xag1758	2-dehydro-3-deoxygalactonokinase (EC 2.7.1.58)	2.15	0
Xag1760	2-dehydro-3-deoxyphosphogalactonate aldolase (EC 4.1.2.21)	2.46	0
Xylose utilization			
Xag1283	β-Xylosidase (EC 3.2.1.37)	0.93	4.45E-81
Xag168	β-Xylosidase (EC 3.2.1.37)	1.52	3.75E-109
Xag1770	Xylulose kinase (EC 2.7.1.17)	-0.91	0
Xag1771	Xylose isomerase (EC 5.3.1.5)	-0.65	1.43E-271
Xag1772	D-xylose proton-symporter XylE	-1.20	0
Xag3800	β-Xylosidase (EC 3.2.1.37)	-1.42	0
Xag3935	β-Xylosidase (EC 3.2.1.37)	0.12	0.002602
Xag3988	Xylose isomerase (EC 5.3.1.5)	-0.81	0
Xag3993	β-Xylosidase (EC 3.2.1.37)	0.11	0.03591
Xag972	Xylanase	3.33	0
Xag973	Xylanase	2.80	0
High affinity phosphate transporter and control of PHO regulon			
Xag1081	Phosphate regulon sensor protein PhoR (SphS) (EC 2.7.13.3)	2.79	0
Xag1082	Phosphate regulon transcriptional regulatory protein PhoB (SphR)	3.12	0
Xag1583	Phosphate ABC transporter, periplasmic phosphate-binding protein PstS (TC 3.A.1.7.1)	5.61	0
Xag1577	Phosphate transport system regulatory protein PhoU	1.44	0
Xag1578	Phosphate transport ATP-binding protein PstB (TC 3.A.1.7.1)	3.72	0
Xag1579	Phosphate transport system permease protein PstA (TC 3.A.1.7.1)	3.38	0
Xag1580	Phosphate transport system permease protein PstC (TC 3.A.1.7.1)	3.08	0
Xag1079	Polyphosphate kinase (EC 2.7.4.1)	1.53	0
Choline and betaine uptake and betaine biosynthesis			
Xag184	L-proline glycine betaine ABC transport system permease protein ProV (TC 3.A.1.12.1)	1.53	0
Xag185	L-proline glycine betaine binding ABC transporter protein ProX (TC 3.A.1.12.1) / Osmotic adaptation	1.64	0
Xag749	Choline dehydrogenase (EC 1.1.99.1)	2.44	0
Xag750	Betaine aldehyde dehydrogenase (EC 1.2.1.8)	2.49	0
Xag751	High-affinity choline uptake protein BetT	3.14	0
Flagellar motility			
Xag1904	Flagellar motor rotation protein MotB	-0.45	2.44E-133
Xag1905	Flagellar motor rotation protein MotA	0.20	1.96E-10

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Table 1. (continued from previous page)

GeneID	Function	Ratio (plant/M9)	
		Fold change (log ₂)	P value
Xag1926	Flagellar synthesis regulator FlhN	-1.31	0
Xag1927	Flagellar biosynthesis protein FlhF	-2.06	0
Xag1928	Flagellar biosynthesis protein FlhF	-1.32	0
Xag1930	Flagellar biosynthesis protein FlhA	-1.55	0
Xag1931	Flagellar biosynthesis protein FlhB	-1.85	0
Xag1935	Flagellar biosynthesis protein FlhR	-2.15	0
Xag1945	Flagellum-specific ATP synthase FliI	-0.68	1.04E-252
Xag1970	Flagellar L-ring protein FlgH	-2.50	0
Xag1974	Flagellar basal-body rod modification protein FlgD	-2.36	0
Xag3475	Flagellar motor rotation protein MotA	0.78	8.13E-134
Xag3476	Flagellar motor rotation protein MotB	1.88	0
Xag1925	RNA polymerase sigma factor for flagellar operon	-0.67	0
Xag1936	Flagellar biosynthesis protein FliQ	-3.02	0
Xag1937	Flagellar biosynthesis protein FliP	-1.98	0
Xag1938	Flagellar biosynthesis protein FliO	-2.14	0
Xag1941	Flagellar biosynthesis protein FliL	-3.42	0
Xag1943	Flagellar hook-length control protein FliK	-0.14	3.82E-12
Xag1944	Flagellar protein FliJ	-0.61	2.33E-157
Xag1946	Flagellar assembly protein FliH	-0.65	3.83E-242
Xag1948	Flagellar M-ring protein FliF	-2.88	0
Xag1949	Flagellar hook-basal body complex protein FliE	-3.22	0
Xag1957	Flagellar regulatory protein FleQ	-0.75	0
Xag1963	Flagellar biosynthesis protein FliS	-0.23	2.82E-32
Xag1964	Flagellar hook-associated protein FliD	-2.41	0
Xag1965	Flagellar biosynthesis protein FliC	0.84	0
Xag1966	Flagellar hook-associated protein FlgL	-0.56	3.37E-71
Xag1967	Flagellar hook-associated protein FlgK	-1.73	0
Xag1968	Flagellar protein FlgJ [peptidoglycan hydrolase] (EC 3.2.1.-)	-1.34	0
Xag1969	Flagellar P-ring protein FlgI	-1.81	0
Xag1971	Flagellar basal-body rod protein FlgG	-2.58	0
Xag1972	Flagellar basal-body rod protein FlgF	-1.27	0
Xag1973	Flagellar hook protein FlgE	-1.60	0
Xag1975	Flagellar basal-body rod protein FlgC	-1.72	0
Xag1976	Flagellar basal-body rod protein FlgB	-3.67	0
Xag1978	Flagellar basal-body P-ring formation protein FlgA	-2.02	0
Xag1904	Flagellar motor rotation protein MotB	-1.87	7.25E-09
TypeIV pilli			
Xag2757	Twitching motility protein PilT	-1.07	0
Xag3062	Two-component sensor PilS	-0.22	6.01E-12
Xag3063	Type IV fimbriae expression regulatory protein PilR	0.05	0.009653
Xag3064	Type IV fimbrial assembly, ATPase PilB	-1.20	1.92E-261
Xag3065	Type IV fimbrial assembly, ATPase PilB	-1.32	0
Xag3067	Type IV fimbrial assembly protein PilC	-1.61	0
Xag3173	Type IV pilus biogenesis protein PilQ	-0.65	0
Xag3174	Type IV pilus biogenesis protein PilQ	-0.15	1.39E-22
Xag3175	Type IV pilus biogenesis protein PilP	-0.47	8.56E-265
Xag3176	Type IV pilus biogenesis protein PilO	-0.49	0
Xag3177	Type IV pilus biogenesis protein PilN	-1.26	0
Xag3178	Type IV pilus biogenesis protein PilM	-0.83	0
Xag3068	Leader peptidase (prepilin peptidase) (EC 3.4.23.43)/N-methyltransferase (EC 2.1.1.-)	-1.19	0

variety of living organisms (Lu and Holmgren 2014). The genes encoding peroxiredoxins (XagID1161) and bacterioferritin (XagID1160) as well as those for several general stress response proteins that might protect *X. axonopodis* pv. *glycines* from oxidative stress at the infection sites were significantly induced in planta.

The expression of *X. axonopodis* pv. *glycines* genes encoding choline dehydrogenase, betaine aldehyde dehydrogenase, and the high-affinity choline uptake protein BetT (XagID749-751) in *X. axonopodis* pv. *glycines* were significantly induced in the soybean apoplast. The genes involved in betaine catabolism and trehalose biosynthesis in *P. syringae* pv. *syringae* have recently been shown to be induced within plants (Yu et al. 2013). These genes also have been reported to play a role in osmotolerance and in fitness on bean leaves (Li et al. 2013). The trehalose biosynthesis genes of *X. axonopodis* pv. *glycines* were also

induced in planta. Trehalose is a common metabolite that has been shown to be involved in conferring tolerance to a variety of environmental stresses, such as low water availability due to high salinity or desiccation, freezing, heat, and oxidative stress (Freeman et al. 2010; Li et al. 2013; Yu et al. 2013), suggesting that *X. axonopodis* pv. *glycines* may experience relatively low water availability in the apoplast of soybean during the infection process, a surprising finding also noted in plants infected by *Pseudomonas syringae* (Li et al. 2013; Yu et al. 2013).

A variety of other genes important to behavior and global regulation in *X. axonopodis* pv. *glycines*, including those involved in chemotaxis, a gene encoding an ECF sigma factor, and the gene encoding RpfN, a regulator of diffusible signal factor-mediated quorum sensing system, were also highly expressed in planta. Moreover, various other genes encoding

Fig. 1. A, Expression of genes encoding polygalacturonase, typeIII effector, and HrpD; B, phosphate transporter proteins; C, RND multidrug efflux pumps; and D, choline dehydrogenase, proline iminopeptidase (*pip*), and the adhesin YapH, as determined by quantitative reverse transcription-polymerase chain reaction at various times after infiltration into soybean leaves. The vertical bars represent the standard deviations of the mean ratio of that compared with in vitro conditions. Similar results were obtained in repeated independent experiments.

proteins with unknown function or showing no similarity to other proteins were also induced.

The expression of a variety of genes involved in attachment and motility were down-regulated in *X. axonopodis* pv. *glycines* in planta (Table 1). As do many bacteria, *Xanthomonas* species synthesize both fimbrial and afimbrial adhesins that mediate their attachment to surfaces. Such adhesins can contribute to virulence (Das et al. 2009; Gottig et al. 2009; Ray et al. 2002), but, depending on the particular stage in the infection process, their presence could interfere with active movement or dispersal processes involved in secondary infection events. The fimbrial adhesins of *X. axonopodis* pv. *glycines* include filamentous hemagglutinin-like proteins, such as homologs of the autotransporter adhesin YapH from *Yersinia* spp. and other proteins (XadA and XadB) that are related to YadA from *Yersinia* spp. Fimbrial adhesins include type IV pili and related proteins, such as the type IV pilus secretin PilQ. The genes encoding a putative hemolysin activation protein, sigma-fimbriae tip adhesin, and genes encoding type IV fimbrial biogenesis protein and type IV pilus biogenesis protein were all suppressed in planta. The gene encoding the adhesin YapH (XagID2132) was particularly strongly down-regulated in planta. The *luxR* homolog XagR had been found to modulate the adhesiveness of *X. axonopodis* pv. *glycines* during the infection process by suppressing YapH, apparently in response to the perception of unidentified small molecules released into the apoplast during the latter stages of the infection process (Chatnaparat et al. 2012). This current study, therefore, confirms these earlier observations and suggests that at least some of the cells of *X. axonopodis* pv. *glycines* had

experienced chemical cues from the infected plant that triggered a conversion to a nonadhesive phenotype that presumably would enable its escape from the apoplast to initiate secondary infections. The observation that many *X. axonopodis* pv. *glycines* genes that would contribute to active motility, including those encoding an RNA polymerase sigma factor for flagellar operon flagellar synthesis regulator (XagID1925), flagellar motor proteins (XagID1904-1905), flagellar biosynthesis protein (XagID1926-1941), flagellar hook-associated protein, and flagellar basal-body rod protein (XagID1963-1978) were expressed at lower levels in planta compared with a minimal medium.

Temporal expression pattern of some plant-inducible genes during the infection process.

To better understand the behavior of *X. axonopodis* pv. *glycines* at various stages during the infection process, we investigated the expression of some of the plant-inducible genes, using quantitative RT-PCR. Expression of genes representing various processes, including the T3SS (*hrpD*) and putative type III effector protein (XagID(1)_100), CWDE PG (XagID691), phosphate transporters (XagID1578, XagID1584, XagID3916), RND efflux pumps (XagID343, XagID1501, XagID2689), and genes controlled by XagR (*pip*, *yapH*, and the choline dehydrogenase encoded by XagID749), was assessed at various times in the first 3 days after inoculation of soybean (Fig. 1). Many genes, such as those encoding the T3SS and effector proteins and CWDE (Fig. 1A) as well as phosphate transporters (Fig. 1B) were strongly expressed immediately after infection, while several RND efflux pumps (Fig. 1C) were highly

expressed only 1 day or more after infection. As expected, *pip* was most highly expressed and *yapH* (Fig. 1D) was most strongly repressed 2 days or more after infection, since they are controlled by XagR, a regulator that accumulates primarily at late stages of infection.

Contribution of plant-inducible genes to the virulence of *X. axonopodis* pv. *glycines*.

Although a few of the plant-inducible genes described here, including those encoding the *hrp* cluster, the cell wall-degrading enzyme protease, endoglucanase, and pectate lyase, Pip and YapH contribute to the virulence of *X. axonopodis* pv. *glycines*, the role of genes that were predominantly expressed in planta has not been established. We, thus, disrupted three different RND multidrug efflux pumps (XagID2689, XagID343 and XagID1501), the malonate decarboxylase operon (*mdc*), and the high-affinity phosphate transport operon (*phoU-pstSCAB*) to assess their role in the virulence of *X. axonopodis* pv. *glycines*.

Efflux pumps.

The three different RND multidrug efflux pumps differed greatly in their contribution to the virulence of *X. axonopodis* pv. *glycines*. While the disruption of efflux pump XagID2689 greatly reduced the virulence of *X. axonopodis* pv. *glycines*, disease severity conferred by the knockout mutant of XagID343 was only modestly reduced and that of XagID1501 was similar compared with that of the wild-type strain (Fig. 2A). The population sizes of these knockout mutants were generally directly related to the virulence that they exhibited (Fig. 2B). The population size attained by the XagID2689 knockout mutant was significantly lower than that of the wild-type strain, particularly at early stages of the infection process (Fig. 2B). In

contrast, the population size of the XagID343 mutant was modestly reduced early in the infection process and that of the XagID1501 mutant was similar to that of the wild-type strain (Fig. 2B). It is noteworthy that XagID2689 was apparently expressed at a substantially higher level than the other efflux pumps, and its greater contribution of variance may have been due to its greater potential for removal of toxic plant products from *X. axonopodis* pv. *glycines*. Given the dominant role of XagID2689 in virulence, we explored in more depth the factors leading to its induction and the functions that it conferred that contributed to *X. axonopodis* pv. *glycines* fitness.

Given that the XagID2689 mutant was strongly reduced in virulence to soybean, we tested the hypothesis that its larger role in virulence was due to its dominant role in tolerance of toxic substances. The susceptibility of the three different knockout mutants of the RND efflux pumps was determined by measuring the minimal inhibition concentration (MIC) of various antibiotics and toxins in vitro compared with the parent strain. XagID2689 was the only mutant that exhibited altered susceptibility to the tested compounds, which included the isoflavonoids naringenin, phloretin, and berberine, as well as the antibiotics acriflavin and tetracyclin in vitro (Table 2). Disruption of XagID343 and XagID1501 had no effect on antimicrobial susceptibility. These results suggested that XagID2689 plays a major role in resistance toward soybean phytoalexins in *X. axonopodis* pv. *glycines*, presumably by serving as an export pump, reducing intracellular concentrations to subinhibitory levels.

To determine whether the compounds identified as substrates of XagID2689 also activated its expression, we introduced plasmid pXag2689:GFP, in which the native promoter of *xagID2689* was fused with a promoterless green fluorescent protein (GFP) reporter gene into the wild-type strain, and GFP fluorescence was determined in the presence and the absence of various antimicrobial compounds of plant origin in vitro. The promoter activity of *xagID2689* was most strongly increased in vitro in the presence of naringenin, with phloretin and quercetin also serving as strong inducers of this efflux pump (Fig. 3A). It is noteworthy that the *xagID2689* promoter was not responsive to a variety of other compounds including (+)-catechin, berberine, acriflavin, salicylic acid, ferulic acid, and benzoic acid (Fig. 3A). Moreover, the promoter activity of *xagID2689* was increased in direct proportion to the concentration of naringenin and phloretin (Fig. 3B). The high level of expression of *xagID2689* seen in planta was, therefore, most likely due to the presence of these phytoalexins released during the infection process. As such toxicants would be expected to be generally present in a variety of plants exposed to potential pathogens, such as *X. axonopodis* pv. *glycines*, we addressed the expression of the *xagID2689* promoter after the introduction of *X. axonopodis* pv. *glycines* into both compatible

Fig. 2. A, Incidence of bacterial pustule disease on soybean incited by different strains of *Xanthomonas axonopodis* pv. *glycines* after spray inoculation on susceptible soybean and B, growth of these strains after infiltration into susceptible soybean. The vertical bars represent the standard deviation of the mean.

Table 2. Sensitivity of *Xanthomonas axonopodis* pv. *glycines* mutants to different compounds

Compound	Minimal inhibition concentrations (µg/ml)			
	Wild type	Xag2689-	Xag343-	Xag1501-
Phloretin	250	125	250	250
Naringenin	500	250	500	500
Quercetin	>1,000	>1,000	>1,000	>1,000
±Catechin	>1,000	>1,000	>1,000	>1,000
Berberine	500	250	1,000	>1,000
Acriflavin	25	6.25	25	25
Tetracyclin	100	50	50	50
Erythromycin	25	50	25	25
Ferulic acid	1,000	1,000	1,000	1,000
Spectinomycin	250	500	500	1,000
Salicylic acid	>1,000	1,000	1,000	1,000

and incompatible host plants. The xagID2689 promoter activity, determined by fluorescence intensity of individual cells as determined by microscopy of cells recovered from plants two days after inoculation, was induced in both the susceptible soybean cultivar Spencer as well as the resistant soybean cultivar Williams and the nonhost tomato (Fig. 3C). These results suggest that the RND-type XagID2689 pump is activated by phytoalexins, most likely a variety of isoflavonoids that are produced in response to interaction with the pathogen.

Malonate metabolism.

Since the C3-dicarboxylic acid malonate is abundant in legumes and we found that genes encoding malonate decarboxylase (*mdc*) that presumably enable the consumption of malonate (XagID590-594) to be strongly induced in planta, we tested the model that *X. axonopodis* pv. *glycines* exploits malonate for growth in the apoplast. The ability of a *mdc* mutant to grow in minimal media with malonate as the sole carbon source and in planta was examined. The *mdc* mutant and the wild-type strain were grown in M9 liquid medium containing 20 mM malonate, 10 mM sucrose, 20 mM succinate, or 20 mM malonate, plus either sucrose or succinate. The *mdc* mutants exhibited almost no growth in minimal media with 20 mM malonate as the sole carbon source, while the wild-type strain exhibited substantial growth (Fig. 4A). The growth of the *mdc* mutant could be restored in malonate minimal media by introducing a plasmid carrying the *mdc* operon (Fig. 4A). To test

the assumption that the induction of the malonate decarboxylase operon was, in fact, due to the perception of malonate by *X. axonopodis* pv. *glycines*, we examined the expression of the gene encoding malonate decarboxylase delta subunit (*mdcD* [XagID591]) in *X. axonopodis* pv. *glycines* by RT-PCR in cells grown in the presence or absence of malonate and other carbon sources. *mdcD* expression was strongly induced in the presence of malonate but not by either sucrose or succinate (Fig. 4B). Furthermore, expression of *mdcD* in the presence of malonate was not appreciably suppressed when either 10 mM sucrose or 10 mM succinate was also present, suggesting that expression of malonate catabolism is not strongly susceptible to catabolize repression by other carbon sources that may be preferentially consumed by *X. axonopodis* pv. *glycines*. It thus seems apparent that *X. axonopodis* pv. *glycines* actively consumes malonate in the apoplast of soybean. Interestingly, disruption of the malonate decarboxylase operon led to little reduction in the virulence of *X. axonopodis* pv. *glycines* to soybean (Fig. 2A) and only modest reductions in the population sizes, achieved within the soybean apoplast after infiltration, that were not statistically significant from the wild-type strain (Fig. 2B). The fitness of the *mdc* mutant was very similar to that of the wild-type strain, as no significant change in the ratio of these two strains, originally inoculated at similar proportions into soybean, were observed with time after inoculation (Supplementary Fig. S2). In aggregate, these observations suggest that, although *X. axonopodis* pv. *glycines* consumes malonate in the apoplast of soybean, it is likely only one of several such carbon compounds available, others of which

Fig. 3. A, Green fluorescent protein (GFP) fluorescence of wild-type *Xanthomonas axonopodis* pv. *glycines* harboring a pXag2689:gfp fusion when grown in culture media containing various compounds, **B,** when grown in media containing different concentrations of two phytoalexins, and **C,** when inoculated into soybean and tomato plants. The vertical bars represent the standard deviation of the mean of relative fluorescence.

Fig. 4. A, Growth of the wild-type *Xanthomonas axonopodis* pv. *glycines* strain (dark bars), the malonate decarboxylase mutant (*mdc-*) (light bars) and *mdc* complemented strain (*mdc+*) (intermediate bars) and **B,** expression of the gene encoding malonate decarboxylase Δ subunit (*mdcD* [XagID591]) when grown in M9 minimal media containing different carbon sources, as determined by quantitative reverse transcription-polymerase chain reaction. The vertical bars represent the standard deviation of the mean population size or ratio of gene expression compared with that of cells grown in M9 minimal supplemented with 10 mM sucrose.

may be either more abundant or more readily catabolized by the pathogen. Thus, malonate is apparently only a minor resource for *X. axonopodis* pv. *glycines* growth in soybean.

Phosphorous metabolism.

Given that the transcription of the *pst* operon (*phoU*-*pstSCAB*) and other genes involved in phosphate metabolism and uptake of *X. axonopodis* pv. *glycines* were induced in planta, we investigated the contribution of this operon to its virulence and growth under phosphate limitation conditions. The virulence of a *phoU*-*pstSCAB* deletion mutant of *X. axonopodis* pv. *glycines* was significantly lower than that of the wild-type strain (Fig. 2A). Likewise, this mutant grew much less rapidly in soybean after inoculation, attaining population sizes that were about 100-fold less than that of the wild-type strain at a given sampling time (Fig. 2B). Surprisingly, however, the reduced growth of the *phoU*-*pstSCAB* mutant in planta was not apparently associated with a deficiency in phosphate acquisition. The growth rate of the *phoU*-*pstSCAB* mutant and the wild-type strain were compared in a low phosphate-containing MOPS minimal media with different concentration of added KPO₄. Similar growth rates and cell yields of the mutant and wild-type strains were observed (data not shown). It seems apparent, therefore, that other transporters in *X. axonopodis* pv. *glycines* allowed sufficient phosphate uptake to enable *phoU*-*pstSCAB* mutants to grow in phosphate-limiting minimal medium and that the *phoU*-*pstSCAB* operon plays additional roles in the interactions of *X. axonopodis* pv. *glycines* with soybean.

DISCUSSION

This study of the global gene expression profiles of *X. axonopodis* pv. *glycines* during its infection of soybean has provided a wealth of information about both virulence traits involved in the interaction as well as the chemical and physical environment within the infection court. RNA-Seq proved to be both a practical and sensitive method by which pathogen transcript abundance could be quantified. A hurdle that we successfully overcame was to identify methodologies by which *X. axonopodis* pv. *glycines* cells could be separated from host tissue in a way that both retained their integrity but also minimized their contamination with eukaryotic RNA. Given that about 60% of the total reads of cDNA could be mapped to the *X. axonopodis* pv. *glycines* draft genome, it appears that the method by which pathogen cells could be made to diffuse from infected plant tissue after minimal disruption of the leaf, similar to that used by Yu et al. (2013), would prove useful in the study of other plant-microbe interactions. *X. axonopodis* pv. *glycines* clearly alters expression of a large variety of genes to successfully interact with soybean during the infection process, since at least 823 genes (16% of the total number of genes) of *X. axonopodis* pv. *glycines* were differentially expressed in the soybean apoplast.

RNA-seq analysis reveals that *X. axonopodis* pv. *glycines* activates the T3SS and effector proteins into the host cell. Most pathogenic xanthomonads possess a T3SS to inject effectors into the host cells (Ryan et al. 2011), and T3SS is critical to *X. axonopodis* pv. *glycines* pathogenicity and virulence (Kim et al. 2003). A variety of pathogenicity and virulence genes of plant-pathogenic bacteria have been shown to be induced in planta. For example, the transcription of genes encoding the type III protein secretion system, effector proteins, and phyto-toxins are strongly up-regulated in various species (Boch et al. 2002; Boureau et al. 2002; Jacobs et al. 2012; Tamir-Ariel et al. 2007; Zhao et al. 2005). In some taxa, such as *Xanthomonas* spp., such virulence genes were associated with a PIP box (plant-inducible promoter) that apparently enabled its induction

in planta (Tamir-Ariel et al. 2007). The regulators HrpG, an OmpR family regulator, and HrpX, an AraC-type transcriptional activator, control the expression of the T3SS in several *Xanthomonas* species (Büttner and Bonas 2010; Guo et al. 2011; Kim et al. 2003; Rossier et al. 1999; Wengelnik et al. 1999). In this study, the expression of *hrpX* but not *hrpG* was significantly up-regulated in *X. axonopodis* pv. *glycines* in soybean. This contrasts with observations made with *Xanthomonas campestris* pv. *vesicatoria* carrying either a *hrpG*-gfp or a *hrpX*-gfp reporter gene construct that revealed that expression of *hrpX* was similar to that of *hrpG* at various times after inoculation into tomato leaves (Zhang et al. 2009). HrpG, which is apparently at the top of the T3SS regulatory cascade (Wengelnik et al. 1999), induces expression of *hrpX*, which, in turn, activates transcription of the operons *hrpB* to *hrpF* (Rossier et al. 1999). Given that the transcriptome of *X. axonopodis* pv. *glycines* was analyzed three days after infiltration of cells into soybean, these results suggest that *hrpG*-mediated regulation was still operative at this early stage of infection but that, if it is only transiently expressed, as is apparently the case in *X. campestris* pv. *vesicatoria*, it may not be expressed in later stages of the infection process. Our analysis of transcript abundance by qRT-PCR at various times also revealed that the T3SS structural gene *hrpD* was strongly expressed as early as 3 h after inoculation, suggesting that plant-derived signals induce T3SS genes of *X. axonopodis* pv. *glycines* nearly immediately after they enter the apoplast.

CWDE, such as PG, cellulases, xylanases, and proteases secreted by the T2SS are important virulence determinants of many plant pathogens. Two PG genes of *X. axonopodis* pv. *glycines* were found to be strongly up-regulated at early stages of infection. This observation is consistent with that seen for the expression of PGs of *E. amylovora*, *Ralstonia solanacearum*, and *X. campestris* pv. *campestris* that were strongly induced in planta (Collmer and Keen 1986; Schacht et al. 2011; Zhao et al. 2005). While knockout of genes encoding PGs attenuate the virulence of many bacteria (Liu et al. 2005; Wang et al. 2008), the role that PG plays in the interaction of *X. axonopodis* pv. *glycines* with soybean is unknown, although pectate lyase (*pel*), plays a role in its induction of a hypersensitive response on nonhost plants (Kaewnum et al. 2006). Since the in planta expression of a PG in Xcc8004 was controlled by HrpG and HrpX (Wang et al. 2008), it is noteworthy that we also found that the expression of the *X. axonopodis* pv. *glycines* PG was also up-regulated early in the infection process, as were genes encoding the T3SS. Moreover, two *X. axonopodis* pv. *glycines* xylanases were also up-regulated. Xylanases from *X. campestris* pv. *campestris*, *X. campestris* pv. *vesicatoria*, and *X. oryzae* pv. *oryzae* were shown to contribute to their virulence (Rajeshwari et al. 2005; Sun et al. 2005; Solé et al. 2015; Szczesny et al. 2010). Both PG and xylanase play a role in plant cell-wall degradation and promotes bacterial invasion and, probably, provide carbon resources in the apoplast. These enzymes also can elicit plant defense responses (Beliën et al. 2006; Déjean et al. 2013; Santos et al. 2014). It is possible that *X. axonopodis* pv. *glycines* primarily employs PG and xylanase to degrade the pectin and xylan that are in high abundance in soybean plant cell wall cells to both release nutrients into the apoplast and to facilitate the translocation of effectors into plant cells during the infection process. Further study of the role of these enzymes in *X. axonopodis* pv. *glycines* should prove fruitful.

Other genes identified in this study may point to the various strategies *X. axonopodis* pv. *glycines* uses to gain access to nutrients. Plant inducible genes in other studies have also identified genes that may enable the pathogen to be otherwise compatible with the environment found within the plant, such as those involved in stress tolerance, polysaccharide synthesis, nutrient uptake, amino acid assimilation, and carbon

metabolism (Jacobs et al. 2012; Yu et al. 2013; Zuluaga et al. 2013). Our results showed *X. axonopodis* pv. *glycines* genes involved in malonate metabolism are induced in planta as well as by malonate, indicating that it encounters this organic acid in the apoplast. Malonate is present in large concentrations in all parts of the soybean plant (Stumpf and Burris 1981; Karunakaran et al. 2013) and plays a role in soybean-microbe interactions. For instance, malonate metabolism contributes to the formation of nitrogen-fixing nodules elicited by *Rhizobium leguminosarum* bv. *trifolii* in clover (An et al. 2002). In contrast, mutants of *Sinorhizobium meliloti* and *R. leguminosarum* biovars *viciae* and *trifolii* that could not catabolize malonate did not decrease N₂ fixation in either alfalfa or clover (Chen et al. 2010; Karunakaran et al. 2013). Consistent with such a finding, the *mdc* mutant of *X. axonopodis* pv. *glycines*, which was incapable of malonate catabolism, was not significantly reduced in either its virulence in soybean or in the apoplastic population sizes achieved. Such a finding might be rationalized, given that the genes encoding fructose transport into the cell were also induced in planta, suggesting that *X. axonopodis* pv. *glycines* also experiences this sugar that is likely an abundant resource in the apoplast (Tukey 1970). Sugars such as fructose or sucrose may also be a preferred carbon source for *X. axonopodis* pv. *glycines*, given that a higher number of cells were produced in culture media containing these compounds compared with that in media containing malonate as a sole carbon source (Fig. 4). It is noteworthy, however, that expression of the *mdc* operon was not apparently subject to repression by either sucrose or succinate in media also containing malonate (Fig. 4). These results, therefore, suggest that *X. axonopodis* pv. *glycines* apparently facultatively utilizes malonate as a carbon source.

The upregulation of *X. axonopodis* pv. *glycines* genes involved in compatible solute metabolism in planta suggests that it experiences low water availability in the apoplast. Choline, glycine betaine, and proline can all function as compatible solutes that are important for bacterial osmoadaptation. Multiple transporters mediating osmoregulatory solute accumulation was identified in the genome of *X. axonopodis* pv. *glycines*, and those that contribute to choline uptake and metabolism were significantly induced in planta. However, the expression of genes encoding putative glycine betaine or proline transporters did not differ in planta from that in a minimal medium. These results indicated that choline is probably abundant in soybean leaves and, as shown in *P. syringae* (Chen and Beattie 2008; Chen et al. 2013), is used by *X. axonopodis* pv. *glycines* to tolerate water stress in the apoplast. In addition, we had previously found that choline dehydrogenase and betaine aldehyde dehydrogenase were induced by XagR, a regulator that accumulates only late in the infection process in soybean (Chatnaparat et al. 2012), suggesting that water withholding may be one defense utilized by soybean to limit colonization of *X. axonopodis* pv. *glycines* after infection, similar to that observed in tomato inoculated with *P. syringae* (Beattie 2011; Chen and Beattie 2008).

Several multidrug transporters in *X. axonopodis* pv. *glycines* were induced in planta and, given that such transporters are usually induced by the toxic compounds that they export, suggesting that it experienced such compounds in the apoplast. In plant pathogens, multidrug efflux systems apparently protect against plant toxic compounds (Burse et al. 2004; Palumbo et al. 1998; Pletzer and Weingart 2014b; Ravirala et al. 2007; Stoitsova et al. 2008; Vargas et al. 2011). Isoflavones are particularly abundant in plants in the family *Leguminosae*, especially in soybeans (Cavaliere et al. 2007). While several RND efflux pumps were induced in soybean, the genes encoding several other efflux pumps were not, suggesting that these exporters play distinct roles in *X. axonopodis* pv. *glycines*. It was noteworthy that, while the XagID2689, XagID343, and

XagID1504 efflux pumps were all strongly up-regulated in soybean, only XagID2689 distributed substantially to resistance toward soybean isoflavones and to the virulence of *X. axonopodis* pv. *glycines* (Fig. 2; Table 2). It seems likely that its dominant role in these processes may have been associated with its higher apparent abundance, as evidenced from the substantially higher induced levels of XagID2689 transcript observed compared with that of the other two RND efflux pumps. That is, while all exhibited fourfold or more induction in planta, the higher absolute levels of expression of XagID2689 likely accounted for its higher potential to export toxic substances. These results are consistent with findings in *E. amylovora* revealing that disruption of the RND efflux pump AcrD does not cause hypersusceptibility to antimicrobial compounds and did not exhibit reduced virulence in either apple rootstocks or immature pear fruits (Pletzer and Weingart 2014a and b). The potential for AcrD to contribute to these processes was, however, demonstrated by overexpressing it in an *acrB*-deficient mutant of *E. amylovora*, which led to increased resistance to several antimicrobial compounds (Pletzer and Weingart 2014 a and b). Thus, it is possible that the expression of XagID2689 was sufficiently high in the XagID342 and XagID1501 mutants that their deletion would have had very little effect on antimicrobial susceptibility and virulence. XagID2689 also appears to be relatively promiscuous in its conference of resistance to toxic compounds. Knockout of XagID2689 led to hypersensitivity to berberine and acriflavin, despite the fact that neither of these compounds contributed substantially to its induction (Table 2). Conversely, expression of XagID2689 was induced by compounds such as quercetin, to which it did contribute substantial resistance (Fig. 3). It was, however, noteworthy that expression of XagID2689 was induced by naringenin and phloretin (Fig. 3), flavonoids that would be expected to be found in soybean. In other bacteria, these flavonoids also activate the multidrug efflux pumps AcrAB and MexAB-OprM of *E. amylovora* and *P. syringae* pv. *tomato*, respectively (Burse et al. 2004; Slimestad et al. 2008; Vargas et al. 2011). Given that the RND efflux pumps were all highly induced within 1 to 2 days of inoculation, it appears that flavonoids are either rapidly induced or are constitutively present in soybean.

X. axonopodis pv. *glycines* also apparently experiences low phosphate availability during infection and responds by upregulating the genes involved in phosphate metabolism and transport. Phosphorus is an important element for all life forms and is often a limiting nutrient in natural environments, usually in the form of inorganic phosphate (Pi). Bacterial responses to phosphorus limitation are important fitness determinants in environments (Lamarche et al. 2008). The Pho regulon is a global regulatory circuit involved in bacterial phosphate management and in pathogenesis (Lamarche et al. 2008). The PhoR-PhoB two-component regulatory system appears essential to coordinating the expression of phosphate-specific transporters encoded by *pstSCAB* as well as genes involved in phosphorus metabolism, such as a phytase to achieve phosphorus homeostasis. In several bacteria, the transcription of the *pst* operon is induced under conditions of phosphate starvation as well as in planta (Crépin et al. 2008; Lamarche et al. 2008; Moreira et al. 2015; Yang et al. 2004). In addition, several studies have linked extracellular phosphate (Pi) availability and the Pst system to virulence and survival under stressful conditions (Crépin et al. 2011; Jacobsen et al. 2008; Lamarche et al. 2008; Pratt et al. 2010). For example, responses to Pi limitation stimulated both adherence to surfaces and biofilm formation in *Agrobacterium tumefaciens* (Danhorn et al. 2004; Xu et al. 2012). Disruption of *pstB*, encoding an ABC phosphate transporter in *Xanthomonas citri*, abolished citrus canker symptoms (Moreira et al. 2015). In this study, the

mutation of the *pst* operon also greatly reduced the virulence of *X. axonopodis* pv. *glycines* to soybean and greatly reduced its apoplast growth in this host. These results further support a model that phosphorus is a limiting factor for bacterial growth in plants, although the other possible roles of phosphorus metabolism in virulence warrant further investigation.

X. axonopodis pv. *glycines* appears to take on a nonmotile yet nonadhesive phenotype during the process of infection of soybean. Several factors, such as fimbrial assembly proteins, type IV pili, and the adhesin YapH that are involved in attachment and flagellar motility of *X. axonopodis* pv. *glycines*, were all suppressed in planta. Previous studies had revealed that the downregulation of YapH, which is repressed by XagR, which itself accumulates only late in the infection process, promotes egression of *X. axonopodis* pv. *glycines* from the apoplast onto the leaf surface (Chatnaparat et al. 2012). The findings here that other fimbriae that are also likely involved in attachment are also suppressed in the soybean apoplast further support the model that escape from infected tissue is an important aspect of the epidemiological cycle of *X. axonopodis* pv. *glycines*. *X. axonopodis* pv. *glycines* does not appear to be capable of active movement within the apoplast, given that most genes involved in flagellar function and chemotaxis are suppressed in this habitat. This finding is similar to that observed in *P. syringae* pv. *syringae*, in which flagellar genes were much more highly expressed on leaf surfaces than in the apoplast (Yu et al. 2013). In contrast, genes mediating adhesion and biofilm formation in the vascular pathogen *X. oryzae* pv. *oryzae* were highly expressed in the xylem of rice (Soto-Suárez et al. 2010). Clearly, bacterial pathogens appear to have a variety of different interactions with their host plant and such distinctive behaviors can be ascertained by global in-planta transcriptome analysis.

Global transcriptional profiling of *X. axonopodis* pv. *glycines* in planta has facilitated the discovery of the genetic elements associated with its adaptation to soybean and its traits that contribute to virulence. Many of the nutrient utilization and virulence traits present in *X. axonopodis* pv. *glycines* appear to be driven by the nature of the plant environment, such as malonate, which are abundant in soybean, phosphorus, and water, which are limited in the apoplast and isoflavonoids that accumulate in plants during abiotic stress. In addition, several traits used by *X. axonopodis* pv. *glycines* to promote disease in soybean are related to those of symbiotic bacteria with this leguminous plant, suggesting that they experience a similar environment.

MATERIALS AND METHODS

Bacterial strains, plasmids, and recombinant techniques.

The bacterial strains and plasmids used in this study are described in Table 3. *X. axonopodis* pv. *glycines* 12-2 was cultured at 28°C in Luria Bertani (LB) agar (LA) for routine work or in the minimal medium M9 (Sambrook et al. 1989). *Escherichia coli* strains were routinely grown in LB medium at 37°C. Bacterial growth was monitored by measuring the absorbance of cell suspensions at 600 nm. Primers used in this work are listed in Supplementary Table S2. Antibiotics were added to media at the following concentrations (per milliliter): kanamycin, 50 µg; chephalexin, 50 µg; and gentamycin, 40 µg. All DNA manipulations, including DNA isolation, plasmid extraction, restriction digestion, ligation, and gel electrophoresis were performed as described previously (Sambrook et al. 1989).

Inoculation and enumeration of bacteria in plants.

Cell suspensions of *X. axonopodis* pv. *glycines* ($OD_{600} = 0.2$; approximately 10^8 cells per milliliter) in water containing 0.01% Silwet L-77 were vacuum infiltrated into soybean cv. Spencer (approximately 4 weeks old) and were incubated for 4 days under plant growth lights with a 12-h photoperiod. Bacterial cells from infected soybean were then harvested in an acidic phenol RNA-stabilizing solution (Yu et al. 2013). Approximately 300 infected soybean leaves were cut into squares (approximately 3×3 mm²) directly into 600 ml of the RNA-stabilizing solution and were sonicated for 10 min. The solution was then filtered through sterilized cheesecloth to remove the plant tissue. The filtrate was centrifuged at $11,295 \times g$ for 10 min, and the pellet was suspended in 3 ml of residual supernatant. The pellet suspension was filtered through a 5-µm filter. The bacterial cells were collected by centrifugation at $6,797 \times g$ for 10 min, the residual supernatant was discarded and the pellets were flash frozen and stored at -80°C until the RNA extraction process. Three biological replicates were performed.

mRNA isolation, mRNA sequencing, and gene expression analysis.

Sequencing was performed on mRNA isolated from *X. axonopodis* pv. *glycines* grown in planta and in M9 minimal medium. Total RNA was isolated with TRIzol (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.), using the method of Santiago-Vazquez and associates (2006). Contaminating DNA

Table 3. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristic ^a	Reference or source
<i>Xanthomonas axonopodis</i> pv. <i>glycines</i> 12-2	Wild type, soybean pathogen	Thowthampitak et al. 2008
XagID2689 mutant	Km ^r , XagID2689::Kan, 12-2 derivative	This study
XagID343 mutant	Km ^r , XagID343::Kan, 12-2 derivative	This study
XagID1501 mutant	Km ^r , XagID1501::Kan, 12-2 derivative	This study
<i>mdc</i> mutant	Km ^r , malonate decarboxylase operon (<i>mdc</i>)::Kan, <i>X. axonopodis</i> pv. <i>glycines</i> 12-2 derivative	This study
<i>phoU-pstSCAB</i> mutant	Km ^r , <i>phoU-pstSCAB</i> ::Kan, <i>X. axonopodis</i> pv. <i>glycines</i> 12-2 derivative	This study
Plasmids		
pTok2	ColE1 replicon, suicide plasmid, Tc ^r	Kitten and Willis 1996
pPROBE-KT	Broad host-range plasmid with a transcriptional fusion cassette containing a promoterless <i>gfp</i> reporter gene; Km ^r	Miller et al. 2000
pKD13	FRT-Km ^r -FRT, oriR6K, Ap ^r , Km ^r	Datsenko and Wanner 2000
pBBR1MCS-5	Broad host-range cloning vector, <i>lacZ</i> , Gm ^r	Kovach et al. 1995
pXag2689:GFP	XagID2689 promoter cloned with <i>Bam</i> HI in pPROBE-KT	This study
pXagmdc	<i>mdc</i> operon with native promoter cloned into pBBR1MCS-5 Gm ^r	This study
pTok2::ΔXagID2689	ΔXagID2689::Kan from overlapping PCR cloned into pTok2, Tc ^r , Km ^r	This study
pTok2::ΔXagID343	ΔXagID343::Kan from overlapping PCR cloned into pTok2, Tc ^r , Km ^r	This study
pTok2::ΔXagID1501	ΔXagID1501::Kan from overlapping PCR cloned into pTok2, Tc ^r , Km ^r	This study
pTok2::Δ <i>mdc</i>	Δ <i>mdc</i> ::Kan from overlapping PCR cloned into pTok2, Tc ^r , Km ^r	This study
pTok2::Δ <i>phoU-pstSCAB</i>	Δ <i>phoU-pstSCAB</i> ::Kan from overlapping PCR cloned into pTok2, Tc ^r , Km ^r	This study

^a Km^r, Tc^r, Gm^r, and Ap^r indicate kanamycin, tetracycline, gentamycin, and ampicillin resistance, respectively; FRT = FLP recombination target.

was removed using the Turbo DNA-free kit (Ambion, Austin, TX, U.S.A.). Because the total RNA of *X. axonopodis* pv. *glycines* in-planta samples was contaminated with plant ribosomal RNA, the eukaryotic ribosomal RNA was removed, using a Ribo-Zero rRNA removal kit for plant leaf (Epicentre, Madison, WI, U.S.A.). The ribosomal RNA of bacteria in planta samples was removed with a Ribo-Zero rRNA removal kit for gram-negative bacteria, according to the manufacturer's protocol. mRNA quality was assessed with a 2100 Bioanalyzer (Agilent, Santa Clara, CA, U.S.A.). Enriched mRNA samples were converted into cDNA, libraries were prepared and were sequenced on an Illumina HiSeq 2000 at the QB3-Functional Genomics Laboratory at the University of California, Berkeley. Three replicate samples were assigned distinct bar-coded linkers and were loaded onto one flow cell. All short reads of cDNA sequence were mapped to *X. axonopodis* pv. *glycines* 12-2 open reading frames using Bowtie 0.12.7 (Chatnarat et al. 2012; Langmead et al. 2009). Differential gene expression was analyzed with edgeR, a Bioconductor package (Gentleman et al. 2004; Robinson et al. 2010).

Total RNA isolation and qRT-PCR.

Total RNA for qRT-PCR analysis was extracted using TRIzol (Invitrogen Life Technologies) and contaminated DNA was eliminated using the Turbo DNA-free kit (Ambion). RNA samples were analyzed for quality using a Bio Analyzer 2100 (Agilent). cDNA was generated from 1 µg of RNA, using SuperScript II (Invitrogen Life Technologies) and random hexamers according to the manufacturer's instructions. To confirm that DNA was removed, samples processed in parallel without reverse transcription served as negative controls in qPCR experiments described below. qPCR was performed on 1 µg of the cDNA, using LightCycler FastStart DNA MasterPLUS SYBR Green I (Roche, Indianapolis, IN, U.S.A.) on an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, U.S.A.), following the manufacturer's specifications. Integration host factor A (*ihfA*) DNA was used as an endogenous control gene to normalize gene expression (Champoiseau et al. 2006). Melting curve analysis was used to verify amplification of a single product. The concentration of amplification products from negative controls (RNA samples to which no superscript was added) was undetectable in all cases, indicating a lack of interference from contaminating DNA.

Generation of knockout mutants.

Site-directed deletion of selected plant-inducible genes was accomplished using splice overlap extension mutagenesis (Heckman and Pease 2007). The upstream and downstream regions flanking target genes in *X. axonopodis* pv. *glycines* were amplified using primers unique to these regions, with one containing an extension complementary to a kanamycin-resistance cassette flanked with FLP recombination target (FRT) sites from pKD13 (Datsenko and Wanner 2000). The two amplicons generated contained ends that overlapped those of the resistance cassette, which were then linked with the resistance cassette by overlap extension PCR. The final larger fragment was then cloned into the destination vector pTok2, using the quick ligation protocol (New England Biolabs, Inc., Beverly, MA, U.S.A.), and was introduced into the mobilizing strain *Escherichia coli* S17-1 by transformation and was then transferred to *X. axonopodis* pv. *glycines* via conjugation. The transformants were plated on LB plates containing kanamycin and cephalixin and the resulting mutants that were kanamycin resistant but tetracycline sensitive were selected. Gene disruption was confirmed using PCR, with primers specific to the sequence flanking each gene.

Virulence and bacterial growth in soybean.

The virulence of *X. axonopodis* pv. *glycines* strains was assessed on soybean cv. Spencer, following topical spray application (Kaewnum et al. 2005). Briefly, cell suspensions of a given strain ($OD_{600} = 0.2$; approximately 10^8 cells per milliliter) in 1 mM buffer were sprayed onto leaves of plants (approximately 4 weeks old) maintained in a greenhouse (average temperature about 25°C). At 7 to 10 days after inoculation, disease severity was assessed by counting the number of lesions on each leaf. Three trifoliate leaves, collected from each of the top, middle, and basal portions of three plants from each of five replicate pots, were evaluated for each strain. The experiments were repeated twice with similar results.

For bacterial growth in soybean, cells of a given strain were resuspended in 1 mM buffer to a final density of approximately 10^6 cells per milliliter and were infiltrated under vacuum into soybean plants (3 to 4 weeks old). Leaves were randomly collected from each plant at a given time point after infiltration (three leaves at each sample time for each strain). Samples were macerated in 5 ml (final volume) of 0.1 M potassium phosphate buffer, using a mortar and pestle. The number of viable cells was determined by dilution plating on LA amended with the appropriate antibiotics. Cell numbers were calculated after enumeration of colonies on plates incubated at 28°C for 48 h. The experiments were repeated three times with similar results.

Antimicrobial agent susceptibility tests.

To measure the antimicrobial agent susceptibility of *X. axonopodis* pv. *glycines* strains, the MIC values of compounds were determined in LB medium by a twofold dilution assay. Microtiter plates (96 well), each containing 100 µl of a twofold dilution series of an antimicrobial solution. Then, 100 µl of a suspension of a *X. axonopodis* pv. *glycines* strain (10^8 cells per milliliter) was added to each well and was incubated for 24 h at 28°C. The MIC was determined by observing the lowest concentration of the antimicrobial in which bacterial growth was inhibited by at least 90%. Growth of bacterial strains was examined at $\lambda = 600$ nm using a Synergy 2 plate reader (BioTek, Winooski, VT, U.S.A.). All tests were done in triplicate.

Promoter activity in vitro and in planta.

The promoter-containing sequences upstream of XagID2689 were amplified from genomic DNA of *X. axonopodis* pv. *glycines* using primers 2689PRO-F and 2689PRO-R. Each amplicon was digested with *Bam*HI (New England BioLabs), was purified using a QIAEX II gel extraction kit (Qiagen Inc., Hilden, Germany), and was cloned immediately 5' to the promoterless GFP reporter gene in pPROBE-KT (Miller et al. 2000). The resulting plasmid, designated pXag2689:GFP, was introduced into *X. axonopodis* pv. *glycines* by electroporation with a Bio-Rad GenePulser X cell electroporation system (Hercules, CA, U.S.A.).

To assess responsiveness of the promoter of Xag2689 to antimicrobial compounds, *X. axonopodis* pv. *glycines* harboring pXag2689:GFP was grown in LB supplemented with different antimicrobial agents. GFP fluorescence intensity and the OD_{600} was determined after 24 h of incubation at 28°C, using a Synergy 2 plate reader (BioTek). Relative fluorescence was determined by normalizing the arbitrary fluorescence units to optical density.

To test the promoter activity of XagID2689 in planta, a *X. axonopodis* pv. *glycines* strain containing pXag2689:GFP was inoculated at 10^8 CFU per milliliter in soybean cultivars Spencer and Williams 82 and in tomato. The GFP fluorescence of bacteria that dispersed from cut leaves at 48 h after inoculation was quantified by epifluorescence microscopy, as in other studies (Burch et al. 2013).

Malonate catabolism.

Consumption of malonate by *X. axonopodis* pv. *glycines* was assessed by adding 100 µl of a suspension (OD₆₀₀ = 0.2) of the wild-type strain or the *mdc* mutant strain to 5 ml of M9 minimal medium supplemented with a given carbon source, and the cell density was measured at 28°C for 24 h.

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