Herbaspirillum rubrisubalbicans as a Phytopathogenic Model to Study the Immune System of *Sorghum bicolor*

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Herbaspirillum rubrisubalbicans is the causal agent of red stripe disease (RSD) and mottle stripe disease of sorghum and sugarcane, respectively. In all, 63 genotypes of *Sorghum bicolor* were inoculated with *H. rubrisubalbicans*, with 59 showing RSD symptoms. Quantitative trait loci (QTL) analysis in a recombinant inbred line (RIL) population identified several QTL associated with variation in resistance to RSD. RNA sequencing analysis identified a number of genes whose transcript levels were differentially regulated during *H. rubrisubalbicans* infection. Among those genes that responded to *H. rubrisubalbicans* inoculation were many involved in plant–pathogen interactions such as leucine-rich repeat receptors, mitogen-activated protein kinase 1, calcium-binding proteins, transcriptional factors (ethylene-responsive element binding factor), and callose synthase. Pretreatment of sorghum leaves with the pathogen-associated molecular pattern (PAMP) molecules flg22 and chitoooligosaccharide provided protection against subsequent challenge with the pathogen, suggesting that PAMP-triggered immunity plays an important role in the sorghum immunity response. These data present baseline information for the use of the genetically tractable *H. rubrisubalbicans*–sorghum pathosystem for the study of innate immunity and disease resistance in this important grain and bioenergy crop. Information gained from the use of this system is likely to be informative for other monocots, including those more intractable for experimental study (e.g., sugarcane).

Keywords: bacterial pathogenesis, defense signaling pathways, elicitors, endophytes, *Herbaspirillum rubrisubalbicans*, MAMPs,

PAMPs, pathosystem, plant–bacteria interaction, resistance factors, *Sorghum bicolor*

Sorghum bicolor is one of the most widely grown crops in the world (Varshney et al. 2012) and has many characteristics that make it an excellent candidate for bioenergy production (McBee et al. 1987; Rooney et al. 2007). Among these characteristics are high yield potential, established production systems, rapid growth, high carbon content, and genetic potential for breeding improvement. Another crucial feature is that sorghum can grow in hot and dry environments and has high water-use efficiency and drought tolerance (Boyer 1982; Rooney et al. 2007; Wiedenfeld 1984). Different microbes can be found in beneficial or pathogenic associations with sorghum. The *Herbaspirillum* genus belongs to the Gram-negative β -Proteobacterium group and includes *Herbaspirillum rubrisubalbicans*, an endophytic, nitrogen-fixing bacterium found in association with several crops such as maize (*Zea mays*), sugarcane (*Saccharum officinarum*), and rice (*Oryza sativa*) (Monteiro et al. 2012a).

Although clearly forming beneficial associations with some plant species, *H. rubrisubalbicans* M1 can also be found in pathogenic association with sorghum and sugarcane (Baldani et al. 1992; Olivares et al. 1996). This bacterium was first isolated from sugarcane (B-4362) leaves in Barbados, where it was shown to be the causative agent of mottle stripe disease and was subsequently shown to cause red stripe disease (RSD) on susceptible sorghum genotypes (Baldani et al. 1992; Galli et al. 1980; Hale and Wilkie 1972; James et al. 1997). The most recent report of a disease caused by *H. rubrisubalbicans* was by Tan and collaborators (2010) in sugarcane in China. RSD can reduce the lifetime of leaves by 50% but does not have the ability to kill the plant and, hence, is generally considered a mild sorghum pathogen (Olivares et al. 1997). *H. rubrisubalbicans* colonizes internal leaf tissues and can also be found in areas of the leaf far from the inoculation point, suggesting free translocation in the host, probably via the vascular system (Baldani et al. 1992; Döbereiner et al. 1993; Olivares et al. 1997). The importance of exopolysaccharides, lipopolysaccharides, and the type 3 secretion system (T3SS) during plant–*H. rubrisubalbicans* association was previously reported

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(Monteiro et al. 2012b; Schmidt et al. 2012; Tuleski et al. 2019) but the specific molecular factors involved in this interaction are still largely unknown. Given that the same bacterial isolate can live in both beneficial and pathogenic association with plants, it would be of interest to define which molecular features define each of these specific lifestyles.

In addition to bacterial factors, the plant immune system may be involved in determining whether *H. rubrisubalbicans* acts as a beneficial or pathogenic microbe. The plant cell has receptors to detect pathogen-associated molecular patterns (PAMPs). These receptors are known as pattern-recognition receptors (PRRs) (Couto and Zipfel 2016) and initiate a process known as PAMP-triggered immunity (PTI) upon recognition of their cognate ligand. The PTI response controls infection before the microorganism colonizes the plant by transcriptional induction of pathogen-responsive genes, production of nitric oxide, reactive oxygen species (ROS), and deposition of callose, among other responses. The net result is that PTI protects the plant against the pathogen (Nürnberger et al. 2004).

Callose is a β -1,3-glucan homo polymer found in association with the cell wall. Callose deposition is induced by multiple stresses in higher plants such as the defense response against biotic and abiotic stress (Chen and Kim 2009). The defense response against pathogen infection occurs through the activation of callose synthase and the formation of a callose synthase complex, which can be modulated by cytosolic Ca^{+2} levels and induction of lipid peroxidation (Chen and Kim 2009; Fredrikson and Larsson 1989; Kauss et al. 1983; Sivaguru et al. 2005; Zavaliev et al. 2011).

In addition to PTI, some pathogenic organisms secrete effector proteins into the host cytoplasm that alter the plant resistance response, facilitating infection. Some effector proteins are themselves recognized (directly or indirectly) by resistance proteins triggering a response called effector-triggered immunity (ETI) (Hogenhout et al. 2009). Because sorghum is now extensively studied as a potential bioenergy crop, which could potentially expand the acreage on which it is grown, it is important to understand and improve the ability of the crop to withstand biotic stress that could significantly reduce yield. In order to do this, it would be advantageous to develop a facile, genetically tractable sorghum pathosystem. Using the *Pseudomonas syringae*–*Arabidopsis thaliana* pathosystem as an example, which has contributed significantly to our general knowledge of plant immunity (Jones and Dangl 2006), we suggest that a bacterial pathogen would be ideal for studies of the sorghum pathogen response. The sorghum–*H. rubrisubalbicans* pathosystem is attractive because both genomes—host and pathogen—have been sequenced; they can be genetically transformed; they are easy to grow under experimental conditions; and they present clear, consistent, and easily measured disease symptoms. Therefore, the interaction of *S. bicolor*–*H. rubrisubalbicans* provides a useful, experimental pathosystem well-suited for studies of disease resistance and innate immunity in this key grain and bioenergy crop.

RESULTS

Screening of *Sorghum bicolor* cultivars for *H. rubrisubalbicans* susceptibility.

Our studies were initiated by first screening 63 genotypes of *Sorghum bicolor* for susceptibility to *H. rubrisubalbicans* strain M1 infection. Disease symptoms were easily observable within 7 days of inoculation. The bacteria were reisolated from the lesions of all genotypes 7 days after inoculation into nitrogen-free NFbHPN-malate medium. The bacteria recovered in this way were subsequently shown to confer disease, fulfilling Koch's postulates.

Of the 63 cultivars initially screened, 59 showed clear disease symptoms in response to *H. rubrisubalbicans* inoculation, with only 4 showing resistance (Supplementary Table S1). The symptoms were characterized by red stripes and points of necrosis along the leaves, as reported by James et al. (1997). The symptoms observed in the 59 genotypes were consistent and exemplified by the BTx623 genotype in Figure 1A.

The appearance of the red stripes and necrotic zones above the point of inoculation suggested active movement of the bacteria within the plant. In order to examine this directly, the level of bacteria within the plant tissues at the site of inoculation and at points above were measured. In each case, infective bacteria could be isolated. All of the susceptible genotypes analyzed showed the same pattern of colonization, as demonstrated by the BTx623 genotype in Figure 1B. The bacterial counts supported the previous hypothesis (James et al. 1997) that high levels of colonization may lead to disease symptom development and necrosis.

Role of stomatal aperture in *H. rubrisubalbicans* infection and possible movement.

Some pathogens can use the stomatal opening to enter their host, even though the plants have developed mechanisms to control this pathway of infection (Melotto et al. 2017). Olivares et al. (1997) and James et al. (1997) reported that the sub-stomatal cavities of sugarcane but not sorghum were colonized by *H. rubrisubalbicans*, suggesting that these could be an entry point.

In order to examine whether stomata may be important to *H. rubrisubalbicans* entry into sorghum and, more specifically, to examine whether the observed movement along the leaf could be due to successive infection of open stomata, plants were treated with abscisic acid (ABA) in order to close the stomata (Albert et al. 2017; Chen et al. 2017), which was confirmed by microscopy (Supplementary Fig. S2A and B), then analyzed for the level of symptoms (Supplementary Fig. S2C and D) and bacterial infection spread (Supplementary Fig. S2E). The results showed that symptom development and bacterial counts were not affected by ABA treatment, indicating that stomatal aperture is not a crucial variable for *H. rubrisubalbicans* movement.

Quantitative trait loci analysis.

H. rubrisubalbicans resistance was analyzed within a recombinant inbred line (RIL) population derived from a cross between a susceptible genotype (BTx623) and a resistant genotype (SC155-14E). In this analysis, resistance was assessed in two ways: direct counting of bacteria colonizing the leaf tissue (Supplementary Fig. S3A) and a visual scale for disease severity using a score of 0 (no disease) to 5 (severe disease with extensive necrosis) (Supplementary Figs. S1 and S3B).

There was substantial variation between the RILs with regard to either the visual severity score or bacterial colonization of leaves. For example, leaf bacterial counts varied from 0 to 10^8 bacteria per gram of fresh leaf, while pathogenicity scores varied from 0 to 4 (Supplementary Fig. S3A and B). According to the Shapiro-Wilk test, the phenotype was not normally distributed in the population (Shapiro and Wilk 1965) and significant differences were identified for all experimental factors (t test), including RIL genotype, experimental run, replication, and the interaction of RIL with replication and experimental run for both visual disease response and bacterial counts (Table 1). Pearson correlation coefficients between replications within each experimental run for visual ratings were high (0.72 to 0.88) and the correlation between experimental runs was 0.64. For bacterial counts, correlation between replications within each experimental run ranged from 0.82 to 0.90 and

between experimental runs was 0.80. A correlation of 0.74 between the visual scoring and bacterial count analyses was observed, indicating that the high number of bacteria is correlated with high levels of pathogenicity.

Quantitative trait loci (QTL) for the visual ratings were detected on chromosomes 3 and 7 within and across experimental runs, with the exceptions that the QTL on chromosome 7 was not detected in the analysis of run 1 and the QTL on chromosome 3 was not detected in the analysis of run 2 (Table 2; only QTL across runs shown). The QTL located on chromosome 3 explained 10.1% and the QTL on chromosome 7 explained 10.4% of the variation in visual disease ratings in the across-runs analysis. The SC155-14E allele at the QTL located on

chromosome 3 increased disease severity by 0.31 on the 0-to-5 visual ratings disease scale and the BTx623 allele for the QTL located on chromosome 7 increased disease severity by 0.32.

QTL for bacterial counts were detected on chromosomes 7 and 10 within and across experimental runs (Table 2; only QTL across runs shown). Two QTL located next to one another on chromosome 7 explained 15.1 and 17.6% of the variation in bacterial counts, and overlapped with QTL detected using visual ratings (Fig. 2). The BTx623 allele for this QTL increased bacterial counts by 0.86 to 0.96 on a scale of 0 to 8.66 (scale based on bacterial counts after log transformation). The QTL located on chromosome 10 explained 20% of the variation in

Fig. 1. Red stripe disease and *Herbaspirillum rubrisubalbicans* M1 colonization in symptomatic leaves. **A**, Typical symptoms of sorghum red stripe disease caused by infection with *H. rubrisubalbicans* M1 in BTx623 sorghum genotype. White arrows indicate necrotic areas. White asterisk indicates red stripe along the leaf. The bacteria were inoculated into the stalk with a hypodermic needle, approximately 1 cm above the soil (not shown on the image). **B**, Counting of total bacteria in the tissue along different points of BTx623 sorghum leaves (refer to A) 7 days after inoculation with *H. rubrisubalbicans* M1 using a hypodermic needle. The count was performed based on the indicated points in A (1 to 4), where 1 is at an initial symptomatic point, 2 is a necrotic point, 3 contains a red stripe, and 4 is an area without symptoms (1 cm above the last symptom area). Bars represent the standard deviation. Assays were performed with three independent replicates and 15 plants per replicate.

Table 1. Analysis of variance significance values from 103 recombinant inbred lines (RILs) evaluated in a greenhouse setting for their *Herbaspirillum rubrisubalbicans* visual disease response and bacterial counts

Source	DF	T3SS ^a	Mean square	F value	Pr > F
Visual disease response					
RIL	105	953.14	9.08	31.28	<0.0001
Replication	2	31.66	15.83	54.55	<0.0001
Run	2	19.03	9.52	32.80	<0.0001
RIL × Replication	210	76.57	0.36	1.26	0.0259
RIL × Run	210	207.19	0.99	3.40	<0.0001
Replication × Run	4	1.24	0.31	1.07	0.3703
Bacterial counts					
RIL	105	5,444.86	51.86	67.73	<0.0001
Replication	2	38.63	19.32	25.23	<0.0001
Run	2	43.68	21.84	28.52	<0.0001
RIL × Replication	210	205.27	0.98	1.28	0.0187
RIL × Run	210	553.47	2.64	3.44	<0.0001
Replication × Run	4	4.72	1.18	1.54	0.1896

^a Type 3 secretion system.

bacterial counts. The SC155-14E allele for this QTL increased bacterial counts by 1.18.

Sorghum gene expression in response to *H. rubrisubalbicans* inoculation.

In order to better understand the sorghum response to *H. rubrisubalbicans* M1 infection, as well as relate gene expression to the QTL data, an RNA sequencing (RNA-seq) analysis was performed using RNA derived from inoculated and uninoculated leaves. The *H. rubrisubalbicans*-susceptible BR007A sorghum genotype was used because it is susceptible to RSD and seed were available in sufficient quantity for the RNA-seq experiments. The plants were grown in vermiculite for 7 days and then inoculated into the stalk using a hypodermic

syringe (an inoculant dose of 10⁷ bacteria). Seven days after inoculation, leaves of 20 plants per triplicate showing symptoms of RSD were harvested for RNA extraction. RNA was also isolated from an equivalent number of leaves from mock-inoculated plants. Mock inoculation was performed in a similar fashion but using saline solution (150 mM NaCl). Hence, the comparison was between mock-inoculated plants and pathogen-inoculated plants. The number and average size of reads for each replicate are shown in Supplementary Table S2. The percentage of reads mapping to ribosomal RNA, mitochondria, chloroplast, and the sorghum chromosomes is shown in Table 3.

The Pearson’s correlation coefficient between the replicates was *R*² = 0.96 and between the treatments was *R*² = 0.94.

Table 2. Parameters of quantitative trait loci (QTL) detected using WinQTL cartographer for *Herbaspirillum rubrisubalbicans* visual disease response and bacterial counts across three experimental runs in a BTx623 × SC155-14E recombinant inbred line mapping population^a

QTL	Parameter ^b	Chr	Peak position (cM)	Peak position (bp)	LOD	Additive effect	<i>R</i> ²	Support interval (cM) 2 LOD	Support interval (bp) 2 LOD
1	VDR	3	44.81	8,313,065	2.65	−0.31	0.1015	41.9–50.0	7,660,601–9,976,473
2	VDR	7	69.41	58177830	2.67	0.32	0.1040	59.1–79.7	52,889,017–89,787,318
1	BC	7	69.41	58,177,830	5.11	0.96	0.1759	65.7–73.9	57,112,406–58,841,459
2	BC	7	77.61	59,470,024	4.49	0.86	0.1511	73.9–79.6	58,841,459–59,787,247
3	BC	10	36.21	4,234,323	5.57	−1.18	0.2007	27.1–38.5	2,897,335–4,740,139

^a Chr = chromosome, cM = centimorgan, and LOD = logarithm of the odds.

^b VDR = visual disease response and BC = bacterial count.

Fig. 2. Curves of logarithm of the odds (LOD) ratio values (upper graph) and additive effects (lower graph) of quantitative trait loci detected using the visual disease response (green line) and bacterial counts (red line) in a BTx623 × SC155-14E recombinant inbred line mapping population. The horizontal line indicates the significance threshold established according to the permutation tests at a significance level of 0.05.

Cluster and principal component analysis (Supplementary Fig. S4) showed good resolution of the data. For further analyses, genes with reads per kilobase per million mapped reads (RPKM) ≥ 1 (approximately 6,000 genes) were considered. In total, 1,049 differentially expressed genes (DEGs) were identified by DESeq (adjusted P [P_{adj}] ≤ 0.05), with 598 upregulated and 451 downregulated (Supplementary Table S3).

In order to further characterize the *H. rubrisubalbicans*–sorghum pathosystem, the list of DEGs was searched with a specific focus on those genes related to pathogen response (Supplementary Table S4). As can be seen from this list, as well as related publications, a variety of important pathogen response genes are regulated in sorghum upon bacterial infection. Examples include pathogenesis-related (PR) proteins, calcium-binding proteins, kinase receptors such as mitogen-activated protein kinase 1 (MPK1), and RIN4.

Several receptors were previously associated with pathogen recognition. The RNA-seq analyses indicated possible PRR homologs differentially expressed during infection, including eight probable leucine-rich repeat (LRR) receptors upregulated and four repressed, as well as a highly repressed gene (111.8-fold)

SORBIDRAFT_09g008890, which encodes for a hypothetical protein with an LRR domain (Table 4). Two wall-associated kinases (WAK), *SORBIDRAFT_02g035920* (9.6-fold) and *SORBIDRAFT_08g020880* (6.7-fold), were also induced by infection with *H. rubrisubalbicans*. Some of these genes were previously shown to be regulated during fungal infection of sorghum (Hipskind et al. 1996; Yazawa et al. 2013). Among other potential pathogen-responsive receptors were six genes homologs of the BAK receptor (Table 4).

Mitogen-activated protein kinases (MAPK) are also responsive to pathogen infection. Anderson et al. (2011) showed that *A. thaliana* MAPK1 acts as a negative regulator of PAMP responses and its inactivation caused increased resistance to bacterial infection. Interestingly, our data showed that a gene (*SORBIDRAFT_04g003480*) encoding a hypothetical protein with 88% identity to *A. thaliana* MPK1 was strongly activated (91.4-fold) by *H. rubrisubalbicans* infection and may also be involved in modulating PTI.

The expression of *SORBIDRAFT_02g002150* was 7.3-fold upregulated in inoculated leaves. This gene encodes a protein with homology to PR1, a class of proteins activated by the

Table 3. RNA sequencing reads mapped against the *Sorghum bicolor* genome

Plants	Replicate 1 (%)	Replicate 2 (%)	Replicate 3 (%)
Mock inoculated			
Total reads after trimming	21,266,660 (100)	10,329,559 (100)	18,767,322 (100)
Ribosomal RNA	6,373,877 (29.97)	3,015,071 (29.19)	4,775,149 (25.44)
Mitochondria	73,068 (0.34)	31,507 (0.31)	69,019 (0.37)
Chloroplast	1,116,362 (5.25)	492,985 (4.77)	1,007,489 (5.37)
Sorghum chromosomes	10,648,598 (50.07)	5,220,611 (50.54)	10,282,440 (54.79)
M1 inoculated			
Total reads after trimming	23,326,723 (100)	17,563,323 (100)	16,012,630 (100)
Ribosomal RNA	5,054,653 (21.66)	3,000,343 (17.08)	2,806,366 (17.52)
Mitochondria	102,830 (0.44)	76,968 (0.44)	70,732 (0.44)
Chloroplast	834,999 (3.58)	692,386 (3.94)	582,687 (3.64)
Sorghum chromosomes	13,302,357 (57.02)	10,611,434 (60.41)	9,874,134 (61.66)

Table 4. Potential sorghum receptor and signaling genes whose expression was modulated by pathogen *Herbaspirillum rubrisubalbicans* M1 infection^a

Gene ID	Locus tag	Probable function	FC	P_{adj}
LRR receptors				
<i>SORBIDRAFT_09g023620</i>	SORBI_3009G175600	Probable LRR receptor-like serine/threonine-protein kinase At1g06840	2.2	5.98E-03
<i>SORBIDRAFT_06g028450</i>	SORBI_3006G214300	Putative LRR-containing protein DDB_G0290503	2.3	5.81E-09
<i>SORBIDRAFT_05g006860</i>	SORBI_3005G084100	Probably inactive LRR receptor-like protein kinase At5g48380	2.4	1.29E-02
<i>SORBIDRAFT_05g026040</i>	...	LRR receptor-like protein kinase	3.4	6.29E-10
<i>SORBIDRAFT_04g011060</i>	SORBI_3004G128200	Probable LRR receptor-like serine/threonine-protein kinase At5g10290	4.5	3.02E-02
<i>SORBIDRAFT_08g006710</i>	SORBI_3008G074500	Probable LRR receptor-like protein kinase At5g49770	5.5	4.70E-04
<i>SORBIDRAFT_09g026090</i>	SORBI_3009G203600	Receptor-like protein kinase HSL1	7.9	2.16E-13
<i>SORBIDRAFT_05g018800</i>	SORBI_3005G126200	LRR protein 1	10.5	4.14E-21
<i>SORBIDRAFT_07g000715</i>	...	LRR protein	-1.4	2.95E-02
<i>SORBIDRAFT_10g010660</i>	SORBI_3010G120800	LRR receptor-like serine/threonine/tyrosine-protein kinase	-2.8	2.70E-05
<i>SORBIDRAFT_01g044540</i>	SORBI_3001G475400	LRR receptor	-3.0	1.19E-03
<i>SORBIDRAFT_09g008890</i>	...	LRR N-terminal domain	-111.8	3.17E-04
RLK				
<i>SORBIDRAFT_01g020950</i>	...	RLK	2.1	8.76E-04
<i>SORBIDRAFT_01g034570</i>	SORBI_3001G362001	Probable serine/threonine-protein kinase PBL15	2.3	4.46E-04
<i>SORBIDRAFT_06g002210</i>	...	Plant RLK	2.5	7.24E-03
<i>SORBIDRAFT_10g006630</i>	SORBI_3010G076600	Serine/threonine-protein kinase RIPK	-5.7	3.60E-02
<i>SORBIDRAFT_01g045010</i>	SORBI_3001G480800	Protein strubbelig-receptor family 7	-6.3	1.55E-06
<i>SORBIDRAFT_08g002100</i>	...	RLK	-10.6	8.61E-03
Calcium signaling pathway				
<i>SORBIDRAFT_02g009790</i>	SORBI_3002G114800	CDPK-18	4.5	5.78E-03
<i>SORBIDRAFT_01g027530</i>	SORBI_3001G279900	CDPK; calmodulin-binding receptor-like cytoplasmic kinase 3	2.5	1.29E-03
<i>SORBIDRAFT_03g011880</i>	SORBI_3003G139500	CDPK; CBL-interacting protein kinase 1	-1.9	1.29E-03
<i>SORBIDRAFT_01g034520</i>	SORBI_3001G361300	CDPK; calcium/calmodulin-dependent serine/threonine-protein kinase 1	-2.7	4.00E-02
<i>SORBIDRAFT_04g005520</i>	SORBI_3004G065900	CML with WRKY-binding domain	-3.1	2.11E-08
<i>SORBIDRAFT_02g024550</i>	SORBI_3002G199500	CaM, probable calcium-binding protein CML45	-3.3	2.27E-08
<i>SORBIDRAFT_08g001200</i>	...	CML with WRKY-binding domain	-13.9	3.34E-02

^a FC = fold change, P_{adj} = adjusted P , LRR = leucine-rich repeat, RLK = receptor-like kinase, CDPK = calcium-dependent protein kinase, CML = calmodulin-like protein, and CaM = calmodulin.

WRKY family of transcriptional factors (TFs) during PTI, impairing the growth and propagation of pathogens in planta (Pajerowska-Mukhtar et al. 2013). Another five WRKY TFs were differentially regulated, including *SORBIDRAFT_03g034670* (2.7-fold induced), *SORBIDRAFT_08g005080* (1.8-fold repressed), *SORBIDRAFT_02g022290* (2.6-fold repressed), *SORBIDRAFT_04g005520* (3.1-fold repressed), and *SORBIDRAFT_03g038510* (3.7-fold repressed). Interestingly, the expression of the last four genes was previously shown to be induced during fungal infection (Yazawa et al. 2013) but our data show them to be repressed by *H. rubrisubalbicans* M1 infection.

The sorghum genome encodes five putative callose synthases (Zhang et al. 2015). One of them, *SORBIDRAFT_01g048630*, with 95% identity with a callose synthase 3-like gene from *Z. mays*, was upregulated 1.9-fold during infection. This protein has a β -1,3-glucan synthase domain and a transmembrane FKS1 domain, normally found in callose synthases. Together with other proteins, the callose complex is associated with the plasma membrane, exporting callose and promoting extracellular accumulation, which is thought to limit pathogen spread.

Another two genes encoding proteins with high identity with *Z. mays* callose synthase were also highly expressed after infection: *SORBIDRAFT_03g034880* (98% identity with callose

synthase 11, upregulated 13.6-fold, *Padj* = 0.08) and *SORBIDRAFT_04g038510* (97% identity with callose synthase-3 like, upregulated 95.1-fold, *Padj* = 0.08).

The presence of upregulated callose synthase and two more enzymes from the same family with high expression suggest that this pathway could be of importance in the sorghum response against *H. rubrisubalbicans*. Callose accumulation upon bacterial infection was confirmed by microscopy analysis (Fig. 3A to D).

The gene *SORBIDRAFT_03g045460*, predicted to encode a protein with a β -1,3-glucan hydrolase conserved domain, was upregulated 5.8-fold. This protein belongs to the PR2 family of β -1,3-glucan hydrolases, capable of degrading fungal cell walls (Kauffmann et al. 1987). Zhang et al. (2015) also implicated these enzymes as components of callose metabolism in sorghum, being responsible for the degradation of callose.

The *H. rubrisubalbicans* genome encodes a T3SS and is likely to produce effectors that potentially could trigger ETI. As a possible response to these effectors, the expression of two RIN4 homologs, *SORBIDRAFT_06g012960* and *SORBIDRAFT_10g010690*, was induced 3.1-fold and 1.7-fold, respectively. In addition, expression of *SORBIDRAFT_10g030240* (heat shock protein 90 [HSP90] homolog) was repressed 2.1-fold. HSP90

Fig. 3. Callose deposition in sorghum BTx623 plants after *Herbaspirillum rubrisubalbicans* M1 inoculation. **A** and **B**, noninoculated leaves and **C** and **D**, leaves 7 days after inoculation with *H. rubrisubalbicans* M1; **E** and **F**, scanned micrograph of the symptomatic area. Loss of tissue and necrotic areas caused by *H. rubrisubalbicans*: **A**, **C**, and **E**, brightfield images; **B**, **D**, and **F**, fluorescent images, which show callose after aniline blue staining. An asterisk (*) indicates red stripe symptoms. **G** to **J**, Callose deposition in 14-day-old plants infiltrated individually with **B**, saline solution; **H**, chitooctaoase; **I**, flg22; and **J**, *H. rubrisubalbicans* M1 and observed using a fluorescence microscope 24 h after infiltration. Fluorescence indicates callose accumulation between the cells.

proteins were previously shown to be important for the function of many plant resistance genes during ETI (Wang et al. 2011).

Three genes encoding calmodulins were repressed by the presence of *H. rubrisubalbicans*, two of them containing a WRKY-binding domain. Also, the analysis indicated that two more Ca^{2+} -dependent protein kinases were upregulated by bacterial infection and two were repressed. The expression of these genes was also shown to be affected by fungal infection (Yazawa et al. 2013). The calcium-signaling pathway is associated with the innate immune system and can be modulated by PAMPs and alterations of intracellular calcium levels. The repression and induction of such genes implies that the bacteria have the ability to modulate the plant immune response to increase susceptibility.

The average reads per kilobase of exons was 11.2 to 12.2 reads/kb. The same analysis in the QTL-specific regions showed an average of 40.4 to 43.3 reads/kb. The results indicate a strong correlation between the RNA-seq and QTL data showing that the QTL regions are, in fact, important and present a higher pathogen-responsive transcriptional response, relative to the global genome. Thirty-six of the differentially regulated genes based on RNA-seq analysis fell within the genome regions defined by QTL analysis (Supplementary Table S5).

The QTL region on chromosome 3 encompasses 210 genes, 50 (23.8%) of which had an RPKM ≥ 0.5 and include 5 genes differentially regulated upon *H. rubrisubalbicans* inoculation. The gene *SORBIDRAFT_03g007910* was repressed 9.1-fold during the development of the RSD and is predicted to encode a protein with conserved domains related to polysaccharide biosynthesis. The gene *SORBIDRAFT_03g008950* was upregulated 2.6-fold and is predicted to encode a nodulin-related protein 1. The biochemical role of this protein is unknown. However, in nonnodulating plants, this class of proteins is important in solute transport and during the interaction with pathogenic microbes, suggesting a role for solute transport in plant innate immunity and during heat stress, as already reported in *A. thaliana* (Denancé et al. 2014; Fu et al. 2010).

The QTL on chromosome 7 encompass 1,015 genes, of which 253 (24.9%) had an RPKM ≥ 0.5 . Of these, 23 were differentially regulated by pathogen inoculation. Among the downregulated genes, *SORBIDRAFT_07g021760* was 3.4-fold repressed and is predicted to encode a putrescine hydroxycinnamoyltransferase 1. These proteins are involved in synthesis of lignin and secondary phenolic compounds, which are known to be induced under stress conditions such as copper stress (Leng et al. 2015). A gene encoding a similar protein was also identified in a QTL analysis of *Picea abies* (Norway spruce) challenged with the pathogenic fungus *Heterobasidion parviporum* (Lind et al. 2014). Also, in maize, the same class of proteins was identified interacting and regulating nucleotide-binding site LRR-resistant proteins, acting as cofactor rather than an enzyme, and finally impairing the hypersensitive response (HR) (Wang et al. 2016).

SORBIDRAFT_07g024290 was downregulated 153.8-fold during infection. This gene encodes a probable alcohol-forming fatty acyl-CoA reductase related to cutin, suberin, and wax biosynthesis, structural wall components important in the defense against biotic and abiotic stress. The modulation of this pathway may indicate that the bacteria can induce morphological alterations to improve their colonization, both to physically facilitate the infection and to decrease the presence of molecules that can activate PTI such as degradation products of the plant's own cuticle through the WAK receptors.

The identified QTL region on chromosome 10 encompasses 248 predicted genes, 68 of which (27.42%) show RPKM ≥ 0.5 . Of these, eight genes were differentially regulated by pathogen inoculation. The gene *SORBIDRAFT_10g004580* was

upregulated 11.4-fold and is predicted to encode an ethylene-responsive element-binding factor.

Activation of PTI protects sorghum from *Herbaspirillum rubrisubalbicans* infection.

As further characterization of the sorghum–*Herbaspirillum rubrisubalbicans* pathosystem, we sought to confirm the importance of PTI in relation to pathogen challenge. In order to test whether PTI could protect sorghum from *H. rubrisubalbicans* infection, the leaves of seven susceptible cultivars were pretreated with flg22 and chitooctase, both well-characterized PAMPs and inducers of PTI, and then challenged with *H. rubrisubalbicans* M1. The goal was to look for alterations in the disease phenotype and callose deposition.

The susceptible genotypes (BTx642, PI506069, PI508366, SCI414, BTx645, LEOTI, and SCI748) were infiltrated with the PAMPs flg22 and chitooctase and, after 2 h, the same location was infiltrated with 10^7 bacteria. Data from separate experiments (not shown), including measurement of ROS, showed that this pretreatment consistently activated PTI. At 7 to 10 days after inoculation, there was significant protection of plant tissues from disease symptoms in the regions where PTI was activated by the PAMP treatment. PAMP pretreatment significantly reduced the size of the lesions in all tested genotypes (Fig. 4A). Pretreatment with flg22 was more effective in most genotypes relative to chitooctase (Fig. 4B).

Fig. 4. Pretreatment with mitogen-activated protein kinases induce protection to the red stripe disease. **A**, Ratio between the symptomatic area of control leaves (inoculated first with a control saline solution and then with *Herbaspirillum rubrisubalbicans*) and leaves first pretreated with pathogen-associated molecular pattern (PAMPs) (i.e., treated with either flg22 or chitooctase [Chi8] and then inoculated with *H. rubrisubalbicans*). **B**, Representative sorghum leaves (BTx623) responding to the various treatments shown in A; that is, those treated first saline solution (Control), 100 nM flg22 (flg22), or 1 μM chitooctase (Chi8) prior to inoculation with *H. rubrisubalbicans* M1. The assay was performed in triplicate, with 20 plants per replicate. Bars indicate the standard deviation.

To further analyze whether treatment with PAMPs was capable of modulating bacterial colonization in the plant, the bacteria were counted in different portions of the leaf (Fig. 5A) from each of the different genotypes, starting at the inoculation point and following the spread of bacteria along the leaf (Fig. 5B; Supplementary Fig. S5).

The number of bacteria decreased significantly as tissues were sampled further away from the inoculation point in control plants. This reduction of total bacterial colonization was pronounced in plants treated with PAMPs prior to inoculation. Also, the difference between the points A, B, and C was significantly reduced in the PAMP-treated plants when compared with the control (Fig. 5B). Interestingly, when chitooctaoase was used, the bacteria were not able to reach point C (2 cm above the inoculation point), which did not occur in the control or in the flg22 treatment. Because flg22 appeared to reduce bacterial numbers at the point of inoculation to a greater extent than chitooctaoase treatment, these data suggest that the effects seen were not strictly due to a reduction of pathogen load but that PTI also appeared to actively suppress movement, presumably within the vascular tissue.

Callose deposition is normally associated with PTI. As mentioned above, genes encoding enzymes associated with callose deposition were among those significantly regulated in response to *H. rubrisubalbicans* inoculation. It was observed

that, during development of RSD, callose accumulated in the leaves 5 to 7 days after infiltration with the bacteria and was concentrated in the symptomatic areas and around necrotic sites (Fig. 3D and F). In order to confirm that callose deposition in sorghum is a response to PAMP treatment, callose deposition was also examined on leaves after treatment with flg22, chitooctaoase, or *H. rubrisubalbicans*. Callose accumulation could be easily seen within 24 h after the PAMP treatments (Fig. 3G to J).

Large amounts of callose deposition between the cells in the flg22- or chitooctaoase-treated plants was observed and this deposition was lighter in plants inoculated only with *H. rubrisubalbicans*. Hence, callose deposition may be one of the factors limiting the spread of the pathogen upon activation of PTI.

DISCUSSION

H. rubrisubalbicans M1 is a bacterium that has a worldwide distribution, having been isolated in several countries, such as Brazil, the United States, and China (Christopher and Edgerton 1930; Tan et al. 2010). Interestingly, many of the sorghum genotypes tested showed a high degree of susceptibility to this pathogen (59 susceptible and 4 resistant).

James et al. (1997) observed that *H. rubrisubalbicans* colonizes the vascular system, leading to the blockage of these vessels and tissue necrosis, likely due to the massive colonization.

Fig. 5. Pretreatment with mitogen-activated protein kinases decreases *Herbaspirillum rubrisubalbicans* leaf colonization. **A**, Image of representative Btx623 sorghum leaves illustrating those areas sampled to generate the data shown in B. Positions A, B, and C refer to the inoculation point, 1 cm above the inoculation point, and 2 cm above the inoculation point, respectively. **B**, Number of bacteria colonizing the internal tissues of sorghum leaves (BTx623) in different tissues around the inoculation point of the control plants (Control), plants pretreated with flg22 (flg22), and plants pretreated with chitooctaoase (Chi8); ND = not detected. An asterisk (*) indicates significant difference at $P \leq 0.05$. The assay was performed in triplicate, with 20 plants per replicate. Bars indicate the standard error.

The necrosis points can be related to the deprivation of nutrients and water caused by the blockage of the vessels, although the production of toxins by the bacterium cannot be ruled out.

Some microbes have the ability to access internal plant tissues by penetrating the leaves or roots through wounds or natural openings such as stomata or pores (Chisholm et al. 2006). In the *H. rubrisubalbicans*–sorghum pathosystem, bacterial spread via the stomata does not appear to be a major route of infection because ABA-induced closure of the stomata did not alter pathogen colonization (Supplementary Fig. S2). These results support the notion that *H. rubrisubalbicans* can penetrate the plant tissue through cuticular or epidermal discontinuities, endophytically colonizing the plant and spreading via the plant vascular system without the need for successive recolonization by epiphytic bacteria (Albert et al. 2017; Chen et al. 2017; Monteiro et al. 2012a; Olivares et al. 1997).

QTL analysis identified several loci associated with variation in resistance to *H. rubrisubalbicans*. A number of genes encoded within the identified QTL regions were shown to be differentially regulated upon pathogen infection and several of these genes are known to play a role in plant immunity (Fig. 6). These genes represent a useful starting point for further characterization of sorghum immunity pathways controlling resistance to *H. rubrisubalbicans*. Such studies should be facilitated by the genetic tractability of both the sorghum host plant and the bacterial pathogen, *H. rubrisubalbicans*. As such, the sorghum–*H. rubrisubalbicans* pathosystem offers many of the same easy tools found in the well-studied *Arabidopsis thaliana*–*Pseudomonas syringae* pathosystem but with the

advantage that such research would involve a crop plant of considerable economic importance.

PAMP pretreatment of sorghum leaves increased the deposition of callose and reduced disease symptoms. Callose deposition promotes cell wall reinforcement that impairs bacterial colonization and subsequent disease development. The sorghum RNA-seq data suggest that callose synthesis and, potentially, degradation are both responsive to pathogen inoculation.

Modulation of PTI by *H. rubrisubalbicans* M1 infection seems to be an important factor in RSD. Bacterial infection affects the expression of several LRR receptor-like kinases, WAK, and MAPK1. QTL analysis identified a region in the sorghum chromosome 7 involved in resistance to RSD, which includes a *cerk1-like* gene (*SORBIDRAFT_07g020210*). CERK1 is a chitin elicitor kinase 1 receptor. This receptor has a well-described role in recognition of fungal pathogens but was also shown in *Arabidopsis* to mediate recognition of peptidoglycan, a component of the bacterial cell wall, which can trigger PTI (Liu et al. 2012; Willmann and Nürnberger 2012). A putative PR1 (*SORBIDRAFT_02g002150*) ortholog was upregulated in inoculated sorghum, which is a gene commonly induced during PTI (Pajerowska-Mukhtar et al. 2013).

Another important plant response to PAMP treatment is callose deposition (Chen and Kim 2009). PAMP treatment of sorghum, as well as inoculation with *H. rubrisubalbicans* M1, induced callose formation. These results were further supported by the fact that genes encoding components of the callose synthase complex were also upregulated upon bacterial inoculation.

Fig. 6. Pathways related to plant pathogen interaction identified as likely responding to *Herbaspirillum rubrisubalbicans* M1 inoculation. CML = calmodulin-like protein, CDPK = calcium-dependent protein kinase, ROS = reactive oxygen species, NOS = nitric-oxide synthase, PTI = pathogen-associated molecular pattern-triggered immunity, LRR = leucine-rich repeat, PGN = peptidoglycan, MAPK = mitogen-activated protein kinase, HSP90 = heat-shock protein 90, and Avr = avirulence.

The *H. rubrisubalbicans* genome encodes a T3SS, which was previously described as critical for development of mottle stripe disease in sugarcane (Schmidt et al. 2011). Hence, it is likely that *H. rubrisubalbicans* uses effector-triggered susceptibility to promote disease development. QTL results identified two candidate genes related to ETI: RIN4 (*SORBIDRAFT_07g026510*) and PtiI (*SORBIDRAFT_07g025590*). In addition, RNA-seq data showed that two RIN4 (*SORBIDRAFT_10g010690* and *SORBIDRAFT_06g012960*) and HSP90 (*SORBIDRAFT_10g030240*) genes are differentially regulated upon pathogen infection. The induction of these genes may reflect an ETI response. Indeed, the necrosis seen in inoculated sorghum is suggestive of an HR commonly associated with ETI (Cui et al. 2015).

The use of model systems is now well established as the most efficient means by which to accelerate research and understanding of complex biological systems. Some associations such as the rice blast fungus *Magnaporthe oryzae* and the maize fungus *Ustilago maydis* have been used to create the basis for our current understanding of plant immunity. Indeed, bacterial pathosystems, especially *A. thaliana*–*P. syringae*, have been crucial to our understanding of plant immunity. The *H. rubrisubalbicans*–sorghum association presents interesting features not found in the *A. thaliana*–*P. syringae* interaction; notably, that *H. rubrisubalbicans*, depending on host genotype, can be either beneficial or pathogenic (Belide et al. 2017; Liu and Godwin 2012; Mathur et al. 2017). The data presented provide a baseline demonstration of the sorghum–*H. rubrisubalbicans* pathosystem, representing both a host and pathogen with completed genome sequences and with well-developed genetic systems. Moreover, *H. rubrisubalbicans* is clearly a mild pathogen that appears to do little economic damage to sorghum production. The examples given in our study suggest a familiar pattern of plant response to bacterial infection (Fig. 6). However, the ability to study *H. rubrisubalbicans* as both a pathogen and a plant-growth-promoting bacterium will hopefully attract researchers interested in understanding the molecular mechanisms used by this organism to support these quite divergent lifestyles. *H. rubrisubalbicans* movement within sorghum leaves may also be a useful system in which to further explore the mechanism of bacterial movement within the plant and how the host may regulate this movement.

MATERIALS AND METHODS

Bacterial strain and growth conditions.

H. rubrisubalbicans M1 was grown in NFbHPN-malate medium (Klassen et al. 1997) at 30°C and 120 rpm for 16 to 24 h in the dark or until an optical density at 600 nm of 1. For plant inoculation, the harvested cells were washed three times with saline solution (150 mM NaCl) to remove all the NFbHPN-malate medium.

Sorghum germination and inoculation.

The surface-sterilized seed (6% hypochlorite for 5 min, 70% ethanol for 5 min, and three washes with sterile water) were planted directly into moist Sunshine mix soil (SunGro Horticulture) and kept covered by a plastic lid in a microenvironment for 5 days until completely germinated. The plants were kept in a greenhouse at 23 to 25°C and were watered every 3 days. After 7 days, the plants were inoculated with 500 µl of saline solution (150 mM NaCl) containing 10⁷ bacterial cells. Inoculation was performed by injection of the solution into the stalk of the plants, approximately 1 cm above the soil with a hypodermic needle. The plants were observed for 5 to 10 days or until development of disease symptoms.

PAMP treatments.

Sorghum plants were grown for 7 days in the greenhouse and then treated with the PAMPs by syringe infiltration with approximately 100 µl of flg22 (100 nM) or chitooctaose (1 µM). After 2 h, the same location was infiltrated with 10⁷ bacteria in 150 mM NaCl. For the control, the plant was infiltrated with saline solution (150 mM NaCl) instead of the PAMP. The plants were observed for 7 to 10 days until the development of disease symptoms.

Image analysis and measurement of lesions.

The measurement of the lesions was performed in the Matlab program using the K-means algorithm. The algorithm was programmed for splitting the picture into three different groups: background, leaf, and symptom. After the first step, the background image was recovered, the borders of the leaves were remodeled, and the necrosis points were extracted and counted as a symptom area. The symptom area was calculated based on the necrotic areas due to the RSD. The ratio between the control leaf symptom area and the treated leaf symptom area was used for the analysis.

Counting of bacteria inside tissues.

A hole punch 0.5 to 0.7 cm in diameter was used to isolate tissues from various sites relative to the site of inoculation. These leaf pieces were washed three times and macerated with saline solution. The solution was serially diluted and plated onto NFbHPN-malate medium (Klassen et al. 1997). The plates were kept in a growth chamber at 30°C.

Callose assay.

Callose detection was performed using aniline blue staining according to Schenk and Schikora (2015). Briefly, the plants were recovered after the treatments and placed in 96% ethanol until total chlorophyll removal (1 to 2 days). The leaves were rehydrated in phosphate solution (150 mM K₂HPO₄) for 30 min and stained with 0.01% aniline blue solution for at least 2 h. The leaves were washed three times with phosphate solution and observed in a fluorescent microscopy with a 4',6-diamidino-2-phenylindole filter (345 nm of excitation).

Role of stomata in bacterial infection.

The plants were grown for 7 days, then infiltrated with 100 µl of ABA. Stomatal aperture was checked before and after the infiltration with ABA by light microscopy. In the same location, 10⁷ bacteria cells were infiltrated. The plants were observed for 7 days after inoculation.

QTL analysis.

Phenotyping. A F_{4:7} biparental mapping population consisting of 103 RILs derived from a cross between the sorghum lines BTx623 and SC155-14E was used.

For each experimental run, plants were arranged in a randomized complete block design with three replications. Two experimental runs were evaluated. After 7 days, the plants were inoculated with 500 µl of saline solution (150 mM NaCl) containing 10⁷ *H. rubrisubalbicans* cells. Inoculation was performed by injection of the solution into the stalk of the plants, approximately 1 cm above the soil with a hypodermic needle. The plants were observed for 5 to 10 days or until the development of the disease.

Two rating systems were utilized to score disease severity: a visual assessment of symptom severity and bacterial counts. The visual assessment was measured on a 0-to-5 scale, where 0 means no symptom and 5 means severe disease symptoms (Supplementary Fig. S1). Bacterial counting from disease tissues was performed as described above.

Data analysis. The normality of data was checked using PROC UNIVARIATE in SAS statistical software, version 9.4 and, subsequently, bacterial counts were log-transformed to achieve normality. An analysis of variance and least square means were generated using the GLM procedure in SAS statistical software, version 9.4, for both visual disease ratings and bacterial counts (Table 1). Standard *F* tests in all analyses were used to determine significance of main effects and interactions. In the model, replications and experimental runs were considered random. Individual hypothesis testing of effects and interactions was designated using an appropriate error term in the GLM procedure. Pearson correlation coefficients were calculated to compare both replications and experimental runs for visual disease ratings and bacterial counts.

QTL mapping.

A genetic linkage map consisting of 956 polymorphic single-nucleotide polymorphisms was obtained from Dr. Patricia Klein at Texas A&M University (Patil et al. 2017). Using Windows QTL cartographer 2.5 (Wang et al. 2012), composite interval mapping was used to identify QTL, estimate QTL logarithm of the odds scores, and measure the proportion of phenotypic variation explained by individual QTL. The walk speed was 1.0 centimorgans, and 1,000 permutation tests at a significance level of 0.05 were performed to simulate estimates for unknown parameters and establish thresholds for the detection of putative QTL (Churchill and Doerge 1994).

RNA-seq analysis.

S. bicolor BR007A plants were grown in vermiculite for 14 days, then inoculated with 10^7 bacterial cells into the stalk. Seven days after inoculation, the leaves were macerated in liquid nitrogen containing Invitrogen RNAlater (Thermo Fisher). The RNA was purified using Trizol (Thermo Fisher Scientific), followed by chloroform extraction and treatment with DNaseI (Thermo Fisher Scientific) according to the manufacturer's instructions. The ribosomal RNA was depleted using the RiboMinus Plant Kit for RNA-Seq (Invitrogen) and the libraries were constructed using the Ion total RNA-seq kit v2 for Whole Transcriptome Library (Life Technologies) following the manufacturer's instructions. Sequencing was performed using the next-generation sequencing platform Ion Proton System (Life Technologies). The Ion PI Template OT2 200 Kit v3 and Ion PI Sequencing 200 Kit v3 (Life Technologies) were used following the manufacturer's instructions. Each treatment was performed in triplicate and each replicate was sequenced at least three times. The mapping was performed using the CLC Workbench Software, version 7.1, using the published *S. bicolor* genome (Paterson et al. 2009) as reference. The genes with RPKM ≥ 1 were considered expressed. The DEGs were identified using the R DESeq package (Anders and Huber 2010).

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