MPMI Vol. 26, No. 7, 2013, pp. 793–800. http://dx.doi.org/10.1094/MPMI-09-12-0208-R.

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Differential Regulation of *Salmonella* Typhimurium Genes Involved in O-Antigen Capsule Production and Their Role in Persistence Within Tomato Fruit

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Submitted 4 September 2012. Accepted 6 March 2013.

Enteric pathogens, including non-typhoidal *Salmonella* spp. and enterovirulent *Escherichia coli*, are capable of persisting and multiplying within plants. Yet, little is still known about the mechanisms of these interactions. This study identified the *Salmonella yihT* gene (involved in synthesis of the O-antigen capsule) as contributing to persistence in immature tomato fruit. Deletion of *yihT* reduced competitive fitness of *S. enterica* sv. Typhimurium in green (but not ripe, regardless of color) tomato fruit by approximately 3 logs. The *yihT* recombinase-based in vivo expression technology (RIVET) reporter was strongly activated in unripe tomato fruit, and fitness of the mutant inversely correlated with the level of the *yihT* gene expression. Expression of *yihT* in mature tomato fruit was low, and *yihT* did not affect competitive fitness within mature fruit. To better understand the molecular basis of the phenotype, behaviors of the *yihT* RIVET reporter and the *yihT* mutant were tested in tomato fruit defective in ethylene signaling. These experiments suggest a role for functional ethylene-mediated signaling in the persistence of *Salmonella* spp. within tomato fruit. Furthermore, jasmonic acid and its precursors strongly reduced expression of *yihT*.

Over the past decade, at least a dozen major outbreaks of salmonellosis have been traced to the consumption of fresh fruit and vegetables (Batz et al. 2011; Mandrell 2009). By some estimates, the burden of the non-typhoidal *Salmonella* infections contracted as a result of raw produce consumption is comparable with the burden of salmonellosis caused by foods of animal origin (Batz et al. 2011). In the aftermath (and, likely, as a consequence) of each produce-linked outbreak of gastroenteritis, the commodity price drops dramatically (Teplitski et al. 2012). Initially, it was hypothesized that these outbreaks are a result of poor sanitation during various production stages. However, produce-linked outbreaks still occur even after significant science-driven improvements in pre- and postharvest handling of fresh produce. It is now well accepted that, under suitable conditions, enteric pathogens are capable of colonizing and persisting within various plant tissues in the field (Danyluk et al. 2008; Greene et al. 2008; Uesugi et al. 2007), possibly as a part of their normal lifecycle (Brandl et al. 2013). Furthermore, growth within plants quickly selects for variants and spontaneous mutants of both *Salmonella enterica* and enterovirulent *Escherichia coli* that are more fit for habitation in plant tissues (Parker et al. 2012; Zaragoza et al. 2012). Despite the apparent importance of the plant-associated lifestyle in the ecology of enterics, relatively little is still known about mechanisms of interactions between enterics and plants, and even less is known about the genetics of *Salmonella*-tomato interactions.

Colonization of tomato surfaces and vegetative and reproductive tissues depends on specific *Salmonella* genes and is a function of the tomato genotype and the physiological state of the plant and specific tissues (Barak and Schroeder 2012; Barak et al. 2011; Noel et al. 2010a; Shi et al. 2007). Approximately 2-log differences were observed in the ability of the same *Salmonella* strain to colonize surfaces of tomato fruit of different species and cultivars (Barak et al. 2011), highlighting the importance of plant genotype in the outcome of plant interactions with enterics. *Salmonella* genetics also contributes to the persistence of the pathogen in plants in general (Tyler and Triplett 2008) and in tomato in particular (Barak and Schroeder 2012; Shi et al. 2007). *Salmonella* spp. horizontally acquired virulence genes do not appear to play a role in persistence of this pathogen within mature tomato fruit (Noel et al. 2010a), suggesting that *Salmonella* spp. use an entirely different set of genes for their interactions with plants (Teplitski et al. 2012).

A screen of *S. enterica* serovar Typhimurium promoters for those differentially regulated within tomato identified approximately 50 unique *Salmonella* genes that were up- or downregulated within red ripe tomato fruit compared with in vitro growth (Noel et al. 2010a). However, none of the corresponding mutations in individual *Salmonella* genes significantly reduced the pathogen's competitive fitness within red ripe tomato fruit. A significant reduction in persistence was only observed after mutations in five of these genes were combined in the same *Salmonella* strain (Noel et al. 2010a), thus suggesting that multiple *Salmonella* sensory, regulatory, and structural inputs contribute to the outcome of the interactions of this bacterium with plants. The *rdar* wild-type phenotype (observed as red, dry wrinkled colonies on low-salt plates containing the Congo Red dye) (Gibson et al. 2006; Romling et al. 2000) strongly correlates with the fitness of *Salmonella* spp. in many

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*The *e*-Xtra logo stands for "electronic extra" and indicates that one supplementary table is published online.

plant interactions (Barak and Schroeder 2012; Brandl et al. 2013; Zaragoza et al. 2012). However, it is not yet certain whether it was the multicellular *rdar* phenotype itself or the regulatory contribution of AfgD, which is one of the many regulators of *rdar* (Gibson et al. 2006; Romling et al. 2000), that was responsible for the increased fitness of *Salmonella* spp. within plants. To address this uncertainty, this study focused on the role of the AgfD-controlled *yih* genes involved in O-antigen capsule synthesis and their role in the persistence of *Salmonella* spp. within tomato fruit.

The *Salmonella* O-antigen capsule is encoded by the divergent operons *yihU-yshA* and *yihV-yihW* (Gibson et al. 2006). These two operons are differently controlled by AgfD, a key regulator of multicellular behavior in *Salmonella* spp., even though they do not contribute to the *rdar* phenotype (Gibson et al. 2006; Romling et al. 2000). None of the genes encoded within the cluster STM4010-STM4030, which includes the *yihU-yshA* and *yihV-yihW* operons, has a consistent fitness defect in the spleen, lung, or liver tissues of mice (even though the mutants are apparently viable under common laboratory conditions because they were present within the tested libraries) (Arrach et al. 2010). Similarly, a screen of 1,045 signature-tagged mutants of serovar Typhimurium in chicks or calves did not identify any of the genes within this cluster (Morgan et al. 2004), although it is not known whether these genes were present in the tested library. A mutation in *yihT* did not affect fitness of serovar Typhimurium in mice infected with the pathogen intraperitoneally (other genes from the *yih* operons were not in the tested libraries) (Santiviago et al. 2009). Collectively, these results suggest that the *yihU-yshA, yihV-yihW* genes are either not involved in virulence or are not expressed inside warm-blooded animals. Consistent with this hypothesis, the *yihU-yshA* putative operon of serovar Enteritidis was not expressed in Luria-Bertani medium (LB) or in human macrophage cells, whereas the divergently encoded *yihVW* genes were co-regulated and expressed at low basal levels in LB and in human macrophages. The predicted *yihX-yihZ* operon was strongly expressed in LB and in macrophages (Ge et al. 2010). Furthermore, none of the promoters of these operons in serovar Typhimurium was identified as differentially regulated in promoter probe screens of genes activated inside mouse tissues (Arrach et al. 2008). Interestingly, the *yihO* gene was identified in a screen of *Salmonella* mutants unable to attach to alfalfa sprouts (Barak et al. 2007). Collectively, these observations suggest that the O-antigen capsule encoded by the *yihU-yshA, yihV-yihW* operons functions in ecological niches outside of warm-blooded hosts, possibly including plants.

This study revealed that *yihT* is differentially regulated in red and green tomato fruit even though no phenotype for the O-antigen capsule mutants was observed in a previous study with red ripe tomato fruit (Noel et al. 2010a). Deletion of the *yihT* gene strongly reduced fitness of *Salmonella* spp. in green tomato fruit as well as in tomato mutants with disrupted ethylene synthesis and signaling. The results offer an important connection between the functions of specific *Salmonella* genes and persistence within plant tissues, and specifically point to plant ethylene-mediated defense and ripening-related pathways as possible candidates for plant processes that facilitate hosting

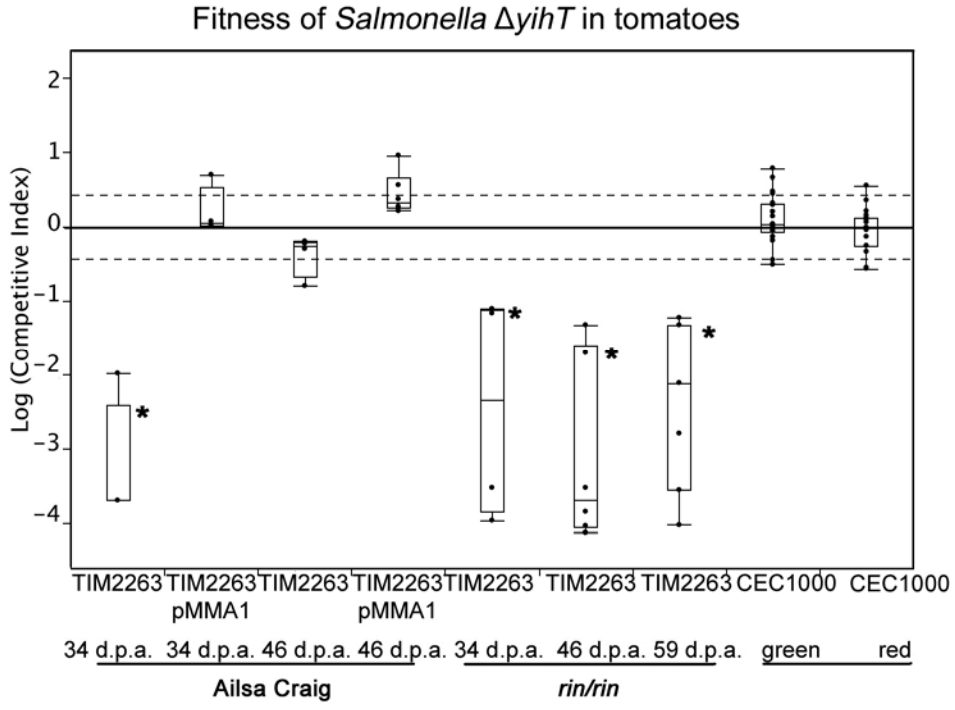

Fig. 1. Competitive fitness of the *yihT* mutant in red and green tomato fruit of 'Ailsa Craig' and *rin/rin* mutant. The *yihT* mutant *Salmonella enterica* sv. Typhimurium TIM2263 (marked with the kanamycin resistance cassette) or complemented strain TIM2263 pMMA1 were co-infected in a 1:1 ratio with the isogenic wild-type serovar Typhimurium 14028 into shallow wounds made in the tomato epidermis with a sterile paper clip. The inoculum dose was estimated by dilution plating to be approximately 50 to 100 CFU/infection. Tomato fruit were incubated for 7 days at 22°C and 41% relative humidity prior to maceration (stomaching) in phosphate-buffered saline and plating onto xylose-lysine deoxycholate medium. Ratios of the wild type to the mutant in the inoculum and in the samples recovered from tomato were estimated by patching on antibiotic-containing plates. Experiments were conducted with at least four biological replicates. Statistical significance was determined by a *t* test against similarly conducted co-infections of serovar Typhimurium 14028 and serovar Typhimurium CEC1000 (marked with a kanamycin-resistance cassette in a presumed neutral site of the genome). Tomato fruit were harvested at the indicated time points (34, 46, or 59 days postanthesis [d.p.a.]) to synchronize developmental stages in tomato fruit, as indicated under the figure. Experiments were conducted in 'Ailsa Craig' or its nearly isogenic *rin/rin* mutant, as indicated in the figure. In box plots, boxes include the lower and upper quartiles, lines within the box are the medians, and whiskers indicate the degree of dispersion of the data. Dashed lines estimate a cut-off beyond which changes in competitive fitness are biologically and statistically significant.

Salmonella spp. and, possibly, other pathogenic or nonpathogenic bacteria.

RESULTS AND DISCUSSION

Deletion of *yihT* strongly reduces fitness in immature tomato fruit.

In our previous experiments with red ripe fruit of 'Campari' tomato, a deletion of *yihT* or the entire *yihT-ompL* cluster did not affect fitness of the mutants (Noel et al. 2010a), even though *yihT* was expressed within tomato fruit (Zaragoza et al. 2012). Consistent with these earlier studies, a deletion of *yihT* did not strongly reduce competitive fitness of the mutant in red (46 days postanthesis [dpa]) 'Ailsa Craig' tomato (Fig. 1). However, competitive fitness of the mutant (measured in the presence of the isogenic wild type strain) in mature green (34 dpa) tomato fruit harvested from the same plant was reduced by 100- to 1,000-fold (Fig. 1). The mutation was fully complemented with a copy of the *yihUT* gene cluster (driven by the native and *lac* promoters) on a low-copy-number vector (Fig. 1). This suggests that the physiological or maturation state of the tissue dramatically affects persistence of *Salmonella* spp. within the fruit. Aside from the implications in food safety, these observations indicate that either *Salmonella* spp. have evolved mechanisms to gauge the suitability of alternate hosts (such as plants) for infection or their plant hosts differentially inhibit endophytic bacterial colonists depending on their developmental or physiological state and in response to the detection of the specific bacterial surface antigens. Therefore, the following experiments focused on defining the behavior of the *yihT* mutant in tomato fruit defective in ripening or pigment production.

Table 1. Competitive fitness of the Δ*yihT* in tomato fruit exposed to ethylene

	Mean of the log competitive index ± SE[a]	
Genotype (dpa)[b]	**No treatment**	**Ethylene treatment**
'Ailsa Craig' (34, green)	–3.272 ± 0.32	–0.465 ± 0.26
'Ailsa Craig' (46, red)	–0.382 ± 0.32	0.600 ± 0.37
rin (34)	–2.445 ± 0.32	–1.142 ± 0.32
rin (46)	–3.098 ± 0.26	–0.440 ± 0.26

[a] SE = standard error. Statistically significant effects ($P < 0.05$) of ethylene treatment are shown in bold.
[b] Tomato genotype at days postanthesis (dpa).

Fitness of *Salmonella* O-antigen capsule mutant in tomato fruit defective in ethylene perception.

In tomato, ethylene-mediated signaling contributes to ripening as well plant defenses. Therefore, the role of the tomato ethylene pathway in the fitness of the *Salmonella* O-antigen capsule mutant was tested. In the tomato *ripening-inhibitor* (*rin*) mutant, a *SEPALATA* MADS-box gene encoding a necessary regulator of fruit ripening is disrupted. *LeMADS-RIN*, a global regulator of ripening, is required to initiate ethylene biosynthesis in addition to ripening factors that cannot be complemented by supplemental ethylene (Martel et al. 2011; Vrebalov et al. 2002). When the *Salmonella yihT* mutant was tested in the tomato *rin/rin* mutant, its fitness was strongly reduced, similarly to its reduced fitness in the green fruit of the nearly isogenic tomato 'Ailsa Craig' (Fig. 1).

The treatment of fruit of 'Ailsa Craig' and *rin/rin* mutants with ethylene alleviated the fitness defect of the *Salmonella yihT* mutant (Table 1) in immature (34 dpa) fruit of 'Ailsa Craig' and

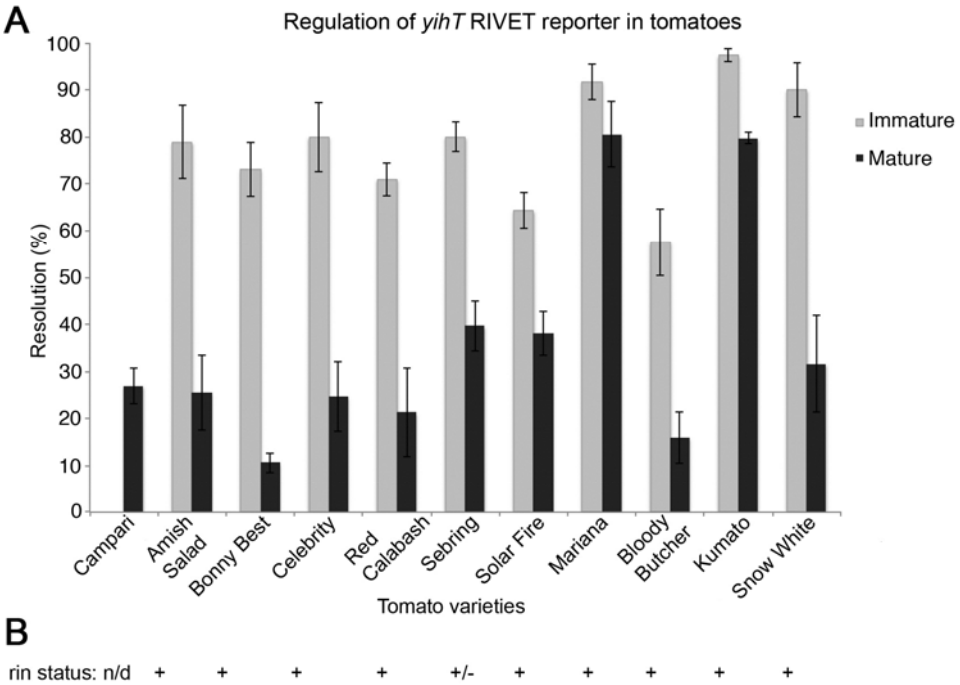

Fig. 2. Resolution of the *yihT* recombinase-based in vivo expression technology (RIVET) reporter in red and green tomato fruit of different commercial varieties. **A,** Cultures of JTN203 were grown overnight from glycerol stock in Luria-Bertani (LB) broth containing tetracycline and washed three times in sterile, deionized water; then, approximately 150 CFU/infection were inoculated into shallow wounds in the tomato epidermis. Following a week-long incubation, each infection was sampled, and colonies formed on xylose-lysine deoxycholate with kanamycin were patched on LB containing tetracycline to determine the resolution of the reporter. 'Campari' tomato fruit (red, unwaxed, sold in a "clam"-type plastic container) were purchased from local supermarkets. All other tomato fruit were grown either under field conditions (conventional in fall 2010 and 2012 in Lake City, FL or transitional organic in spring 2011 and 2012 in Archer, FL) or in a roof-top greenhouse on the University of Florida campus. The *yihT* RIVET reporter was tested in both field- and greenhouse-grown tomato fruit of each variety except for Campari. **B,** Status of the *rin* allele in the tested tomato varieties is shown. 'Sebring' is heterozygous for *rin*, while all other cultivars are homozygous for the wild-type *Rin* allele.

in the *rin/rin* fruit (34 and 46 dpa), and had no effect in mature (46 dpa) fruit of 'Ailsa Craig'. Even though the ethylene-dependent rescue of the fitness of the *yihT* mutant was statistically significant, fitness was not completely restored to the level of the wild type. Because ripening in the *rin/rin* mutant cannot be restored by ethylene, the ability of the exogenously added ethylene to at least partially restore fitness of the *Salmonella yihT* mutant suggests that both *LeMADS-RIN* and ethylene affect the ability of *Salmonella* spp. to persist in tomato fruit.

Regulation of the *Salmonella* O-antigen capsule genes in tomato ripening and ethylene mutants.

To better understand how *Salmonella* O-antigen capsule genes contribute to the ability of this human pathogen to interact with plants, regulation of the *yihT* and *yihX* recombinase-based in vivo expression technology (RIVET) reporters was tested in mature and immature fruit of tomato 'Ailsa Craig' and its isogenic *rin/rin* and *Nr/Nr* mutants at 34 (mature green), 46 (ripe), and 59 dpa. The *Nr* locus encodes an ethylene receptor with impaired ethylene-binding capacity causing delayed and incomplete ripening of the *Nr* mutants due to reduced ethylene sensitivity (Yen et al. 1995). The *rin/rin* and *Nr/Nr* fruit were the same dpa as the wild-type 'Ailsa Craig' control but mature to green and orange, respectively, by 59 days.

In vivo reporters (such as RIVET) have an important advantage for documenting *Salmonella* gene expression within alternate hosts (such as plants) (Teplitski et al. 2012). RIVET is based on the activation of a recombinase (typically, TnpR) cloned downstream of the promoter of interest. TnpR then excises a selectable marker (resistance to tetracycline, for example), and the loss of the marker is then scored quantitatively by patching colonies of bacteria recovered from the host onto plates containing antibiotics (Angelichio and Camilli 2002). RIVET works well in *Salmonella* spp. and is as sensitive as the *lacZ* reporter in vitro; however, there are important caveats in the interpretation of the RIVET data in general (Merighi et al. 2005; Noel et al. 2010b) and as it relates to the *Salmonella* spp.–plant interactions (Teplitski et al. 2012). The measurable resolution of the *yihT* RIVET reporter in soft LB agar is negligible and is increased when the reporter is incubated inside tomato fruit (Zaragoza et al. 2012).

Expression of the *yihT* RIVET reporter in *rin/rin* tomato at all three maturity stages was high, and indistinguishable from the activity of the reporter in immature (34 dpa) fruit of the parental 'Ailsa Craig' (Fig. 2A). In the *Nr/Nr* tomato (reduced response to ethylene), resolution of the *yihT* RIVET reporter was high at 46 days (comparable with mature green, 34 dpa tomato fruit of the parent). In 59-dpa *Nr/Nr* tomato fruit, the resolution of the reporter decreased to the levels comparable with the red ripe (46 days old) wild type. Because these tomato mutants lack ripening ethylene synthesis (*rin*) or are deficient in their ability to respond to it (*Nr*), these observations suggest that the upregulation of *yihT* inside green tomato fruit (or downregulation in red tomato fruit) is a consequence of fruit maturity indicators, possibly including ethylene-dependent changes in tomato defense responses or physiology.

Table 2. Contribution of tomato metabolites to the expression of *yihT* in *Salmonella enterica* JTN203

	Mean *yihT* resolution (%) (± SE)[a]	
Carbon sources[b]	**M9**	**1/10 LB**
H_2O (control)	26 ± 4	5 ± 2
Organic, green[c]	16 ± 2	5 ± 2
Organic, red[d]	9 ± 4	2 ± 1
Glucose ⇑	14 ± 3	8 ± 3
Sucrose ⇓	31 ± 10	3 ± 1
Glycerol	6 ± 4	8 ± 3
Mannitol ⇓	16 ± 4	8 ± 3
Myo-inositol ⇓	16 ± 6	4 ± 1
Arginine ⇓	17 ± 6	5 ± 2
Glycine ⇓	25 ± 4	9 ± 4
Linoleic acid ⇓	1 ± 0.5	1 ± 0.4
Palmitic acid ⇓	26 ± 0.5	5 ± 1
γ-linolenic acid	2 ± 1	2 ± 1
13(S)-HpOTrE(γ)	16 ± 3	5 ± 2
12-*oxo*-phytodienoic acid	10 ± 2	2 ± 1
Jasmonic acid	2 ± 1	1 ± 0.4
Decanoic acid (Control)	1 ± 1	1 ± 0.4

[a] Mean *yihT* reporter resolution ± standard error (SE) and LB = Luria-Bertani medium.

[b] An upward arrow indicates an increase in accumulation of the metabolite in tomato fruit as they mature (Carrari et al. 2006) and a downward arrow indicates that accumulation of the metabolite decreases as the fruit matures. Glycerol is present at the same amount in red and green tomato fruit (Carrari et al. 2006).

[c] Organic extract, 'Ailsa Craig' green tomato, 34 days postanthesis (dpa).

[d] Organic extract, 'Ailsa Craig' red tomato, 46 dpa.

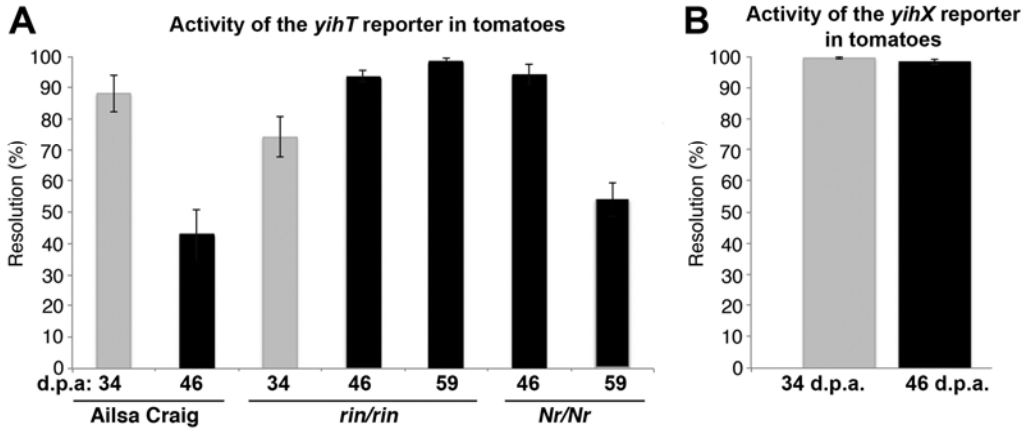

Fig. 3. Resolution of the *yih* recombinase-based in vivo expression technology (RIVET) reporters in tomato 'Ailsa Craig'. **A,** Resolution of the *yihT* RIVET reporter was tested in red ripe (46 days postanthesis [d.p.a.]) and mature green (34 d.p.a.) fruit of 'Ailsa Craig'. Resolution of the reporter was similarly tested in isogenic tomato mutants with defects in ethylene production (*rin/rin*) or impaired response to ethylene (*Nr/Nr*). Because ripening of these mutants is inhibited, neither physiological nor visual assessment of ripening is applicable. Thus, sampling was synchronized to the wild type by tagging tomato fruit and harvesting at the same age as the wild type. Four to six biological replications were carried out and averages of all experiments are shown; error bars are standard deviations. **B,** Resolution of the *yihX* RIVET reporter in green (34 d.p.a.) and red (46 d.p.a.). As a control, the reporter was tested in soft Luria-Bertani agar, and the resolution was found to be 87 ± 3%.

As a control, activity of the *yihX* RIVET reporter was tested under the same conditions. The *yihX* RIVET reporter was strongly expressed in LB (87 ± 3%), consistent with microarray data in serovar Enteritidis (Ge et al. 2010). The *yihX* reporter was expressed in mature and immature tomato of 'Ailsa Craig' (Fig. 3). This suggests that the responses of the *yihT* RIVET reporter observed in this study are specific to this operon and are not artifactual.

Expression of the *yihT* RIVET reporter in commercial tomato varieties.

To follow up on the experiments with the fitness of the *Salmonella yihT* O-antigen capsule mutant in tomato defective in ethylene synthesis and perception, *yihT*-dependent phenotypes were tested in commercial tomato varieties.

The *yihT* RIVET reporter was resolved at 10 to 40% in red ripe tomato fruit of 'Campari', 'Amish Salad', 'Bonny Best', 'Celebrity', 'Red Calabash', 'Sebring', and 'Bloody Butcher' (Fig. 2). A higher level of resolution (approximately 80%) was observed in ripe tomato fruit of 'Mariana' and 'Kumato'. A much higher, and fairly uniform, level of resolution (60 to 100%) was observed in green tomato fruit of all varieties. The presence of pigments per se does not seem to affect the activity of the reporter, because the fruit of Mariana turn red when mature whereas mature fruit of Kumato appear brown (because, in addition to the red pigments, they retain chlorophyll due to the *green-flesh* (*gf*) mutation in a STAY-GREEN protein coding gene) (Barry et al. 2008). However, resolution of the reporter in mature tomato fruit of both of these varieties seems higher than in other tomato fruit that turn red when mature. Therefore, it is reasonable to hypothesize that major pigments associated with ripening did not affect the resolution of the *yihT* RIVET reporter.

Because some commercial tomato varieties are heterozygous for *rin*, tomato varieties in which expression of the *Salmonella yihT* was tested were genotyped. Of the 10 tested varieties, only 'Sebring' was heterozygous for *rin*. Even though expression of *Salmonella yihT* inside red tomato fruit of 'Sebring' was higher than in 'Campari', 'Amish Salad', 'Bonny Best', 'Celebrity', 'Red Calabash', and 'Bloody Butcher', it was the same as in 'Solar Fire' and 'Snow White' and less than in 'Mariana' and 'Kumato' (all four of which carry the wild-type allele of *Rin*) (Fig. 3).

Differences in the resolution of the *yihT* reporter in tomato fruit of 'Kumato', 'Snow White', and 'Mariana' (Fig. 2) prompted us to investigate whether fitness of the mutants in ripe fruit of these varieties is inversely proportional with the level of the *yihT* expression, as has been observed in green fruit and in *rin* tomato fruit (Figs. 1 and 3). The competitive fitness of the *yihT* mutant in ripe tomato fruit of 'Kumato', 'Snow White', and 'Mariana' was –0.75 ± 0.16, 1.24 ± 0.23, and 0.93 ± 0.13, respectively. Even though fitness of the mutant in 'Kumato' tomato was reduced, this reduction was not statistically significant (when compared with co-infections of the wild type and a neutral kanamycin-resistant mutant). In tomato fruit of 'Snow White' and 'Mariana', the mutant was modestly but statistically significantly more fit than the wild type. Thus, the inverse correlation between the level of the *yihT* expression and the fitness of the mutant is consistent in unripe tomato fruit and in the tomato mutants defective in ripening and ethylene signaling. What cues are responsible for the differential regulation of *yihT* in green versus ripe tomato fruit is not yet known.

Contribution of tomato metabolites to the regulation of the *Salmonella yihT* gene.

In an attempt to test whether metabolites or hydrophobic secondary products associated with ripening affect regulation of the *yihT* RIVET reporter, the effect of the hydrophobic extract of red and green tomato fruit ('Ailsa Craig') and commercially available metabolites on the reporter were tested in soft agar, as previously described (Noel et al. 2010a). Assays were conducted in M9 minimal medium an' also in $^{1}/_{10}$ diluted LB broth. In general, resolution of the reporter was lower in $^{1}/_{10}$ LB compared with M9; however, these differences are almost certainly not due to the differences in the growth rates, because the resolution of the RIVET reporter is not affected by growth rates.

Hydrophobic compounds extracted from red or green tomato fruit did not elicit significant responses in the *yihT* reporter, although the extracts of green (34 dpa) fruit of 'Ailsa Craig' were somewhat more stimulatory than the extracts of the red (46 dpa) fruit. However, the resolution of the reporter in response to extracts or pure compounds did not reach levels observed in green tomato fruit (Table 2 versus Figs. 2 and 3). In the presence of linoleic acid, expression of the *yihT* reporter was essentially eliminated (Table 2). To ascertain that linoleic acid affects the reporter rather than the activity of the TnpR resolvase, the *lacZ* activity of the same RIVET reporter was tested and was similarly reduced (data not shown). The role of linoleic acid in the elimination of the expression of *yihT* is surprising, because the amounts of linoleic acid are higher in green tomato fruit (compared with red tomato fruit) and it was linoleic acid that was responsible for the upregulation of *Salmonella fadH* in green tomato fruit (Noel et al. 2010a).

Jasmonic acid and its biosynthetic precursors (γ-linolenic acid, 13(S)-HpOTrE(γ) and 12-oxo-phytodienoic acid) strongly reduced activity of the *yihT* reporter (Table 2). However, this response may be fairly generic because other fatty acids (decanoic and linoleic but not palmitic) affected the reporter (Table 2). It is tempting to speculate that, in response to the detection of the plant defense compounds such as jasmonate (and its precursors) and ethylene, *Salmonella* spp. modify expression of the genes involved in the synthesis of mitogen-associated molecular patterns. However, further evidence is needed in support of this hypothesis.

Evidence for the involvement of a higher-level regulator in controlling *Salmonella* gene regulation in response to tomato ethylene signaling.

The RIVET reporters used in this study are all in structural genes, themselves subject to control by various regulators, including AgfD (Gibson et al. 2006) and RpoS (Zaragoza et al. 2012). Because AgfD is the only identified regulator of the *yih* operons, we tested regulation of another AgfD-dependent gene (*agfB*) in 'Ailsa Craig' tomato fruit and its isogenic *rin/rin* and *Nr/Nr* mutants to establish whether a correlation exists between expression of *yihT* and *agfB* and found a modest (but statistically significant) linear correlation between *yihT* and *agfB* expression under these conditions (Fig. 4).

These observations are intriguing because they suggest that a higher-level regulator, common to at least two genes involved in persistence of *Salmonella* spp. within alternate hosts, is capable of detecting a cue produced by tomato fruit that likely involves an intact ethylene synthesis or response pathway. At this time, neither the sensor nor the cue perceived by it is known.

One may hypothesize that AgfD serves as an integration point of this signaling input. AgfD is a member of the Uhp (FixJ) family of transcriptional regulators which contain a predicted receiver domain (including a conserved phosporylation site) (Romling et al. 2000). A cognate sensor kinase for AgfD is not known and, once identified, the search for the signal or an environmental condition perceived by it can proceed more efficiently. It will likely reveal novel layers of signal exchange between enteric pathogens and their alternate hosts.

MATERIALS AND METHODS

Bacterial strains.

The construction of TIM2263 (Δ*yihT*27::kan) and its derivative JTN203 Δ*yihT*27-*tnpR-lacZ yjeP::res1-tetRA-res1* in *S. enterica* sv. Typhimurium ATCC14028 background has been described previously (Noel et al. 2010a; Zaragoza et al. 2012). *S. enterica* sv. Typhimurium JTN202 (14028 Δ*agfC37-tnpRlacZ yjeP::res1-tetRA-res1*) was constructed previously (Noel et al. 2010a). Because the promoterless *tnpR-lacZ* cassette in this reporter replaces the *agfC* open reading frame within the operon, this reporter is considered to serve as a read-out for the regulation of the *agfB* promoter, which is the first gene of the operon.

The *yihX* RIVET reporter (CEC0023) was constructed essentially as by Osorio and associates (2005). The RIVET reporter was constructed as a merodiploid, in which the promoterless bicistronic *tnpR-lacZ* cassette was cloned downstream from the predicted *yihX* promoter and the wild-type copy of the promoter was reconstituted downstream. A fragment spanning the predicted promoter region was amplified using *S. enterica* sv. Typhimurium 14028 genomic DNA as a template, with primers ctcgagCAACGAAGCCAGCGTAGTGA and ctcgagATCGGCAGCGGAAAAACCTT (introduced *XhoI* sites are shown in lowercase). The fragment amplified with *Taq* polymerase (New England Biolabs, Beverly, MA, U.S.A.) was cloned into pCR2.1 and then released with *XhoI*. The resulting fragment was gel purified and ligated into pGOA1193 (Osorio et al. 2005) linearized with *XhoI* and treated with calf intestinal alkaline phosphatase. The ligation reaction was transformed into *E. coli* DH5α λpir. The presence of the insert was verified by polymerase chain reaction (PCR) and sequencing at the University of Florida Interdisciplinary Center for Biotechnology Research Core Facility (Gainesville, FL, U.S.A.) using primer MT59, which binds within *tnpR* (Noel et al. 2010b). The sequenced plasmid was electroporated into *E. coli* BW20767 and then mated with *S. enterica* sv. Typhimurium JS246 (Merighi et al. 2005). Transconjugants were selected on M9 agar containing 0.4% glucose, ampicillin, tetracycline, and X-gal at 37°C. Individual colonies were restreaked onto fresh plates to purity. Integration of the promoter-*tnpR-lacZ* fusion into the chromosome was confirmed by PCR using primer CTCGTCACCAGCGTAGGC (which binds upstream of the initial forward primer used to amplify the target fragment) and MT59. Once confirmed, the construct was stored in glycerol from an overnight culture in LB with ampicillin and tetracycline.

For fitness tests, a strain of serovar Typhimurium (CEC1000) marked with a kanamycin-resistance cassette in a presumed neutral site of the genome was constructed. The *frt-kan-frt* cassette was inserted in the intergenic region upstream of *phoN* (between STM4318 and STM4319, *phoN*) using Datsenko and Wanner (2000) mutagenesis and primers GCTTCAGCCGGAC AACGACGTCATGACGGTCTGGACGGACTGCAAACGGt gtaggctggagctgcttcg and AGAATGCCTTTGGTTTCCCCGAT TCGCATGAATTCAACGCCCCCTTCCCcatatgaatatcctccttag. The insertion was confirmed with primers GGGAGTTCGGG CAAAGCA and K2 (Datsenko and Wanner 2000).

To complement the *yihT* mutation in *S. enterica* sv. Typhimurium TIM2263, a wild-type copy of the gene with a 552-bp region upstream of *yihUT*, presumed to contain a native promoter, was amplified with Vent *Taq*-catalyzed PCR with primers *ctatgt*ggatccACATCCCACTGCGAAAAATC and *tgacta* aagcttTTAGAGGCATCTACCACGCC and serovar Typhimurium 14028 genomic DNA as a template (sequences in uppercase bind to the *Salmonella* chromosome, *Bam*HI and *Hin*dIII restriction sites are shown in lowercase, and anchor sequences upstream of the restriction sites are indicated by italics). The PCR product was digested with *Bam*HI and *Hin*dIII, then

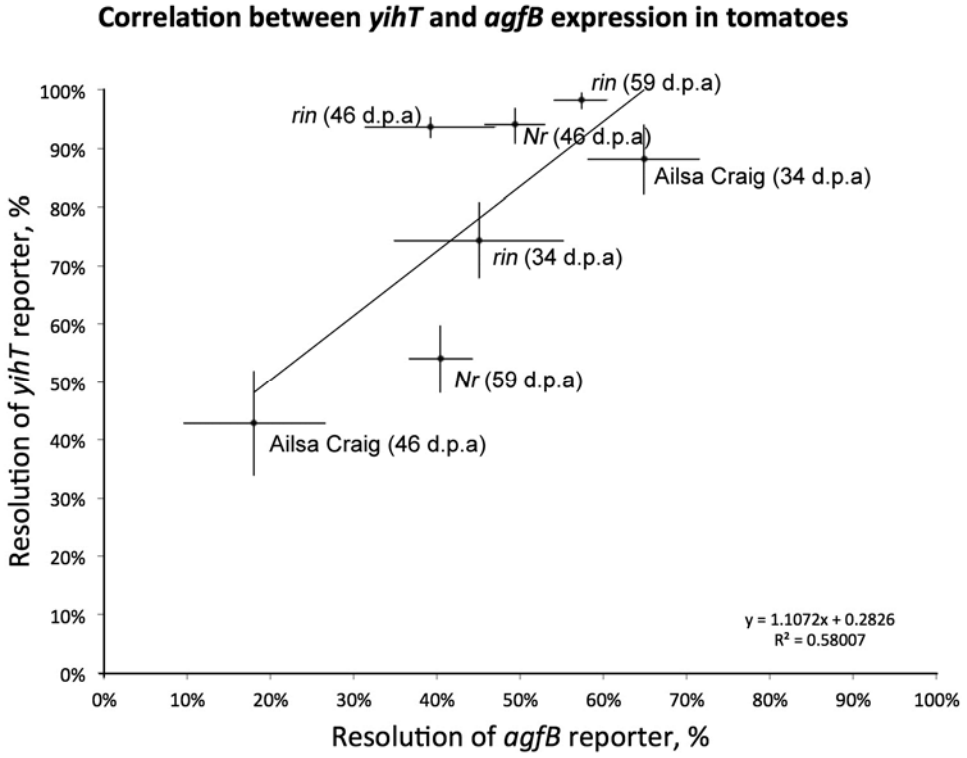

Fig. 4. Co-regulation of the AgfD-regulated recombinase-based in vivo expression technology (RIVET) reporters in tomato. To test whether the tomato environment similarly affects two RIVET reporters, each controlled by the *Salmonella* response regulator AgfD, resolution of JTN203 and JTN202 was tested in tomato fruit of 'Ailsa Craig' and the homozygous *rin* and *Nr* mutants. Tomato fruit were harvested at 34, 46, or 57 days postanthesis (d.p.a.). Each data point represents average resolution of the reporters in a specific tomato mutant or wild type. Error bars are standard deviations of three technical and three biological replications.

cloned into pWSK29 digested with the same enzymes. The ligation reaction was transformed into chemically competent *E. coli* DH5α. The insertion was confirmed by PCR and sequencing at the DNA Lab, Arizona State University. In the resulting plasmid pMMA1, the *yihUT* genes with their own upstream region are cloned downstream from the *lac* promoter (which is not regulated in *Salmonella* spp. and is constitutive) and in the same orientation as the P*lac*. The pMMA1 plasmid was first electroporated into the restriction-minus modification-plus serovar Typhimurium JS198, from which the plasmid was extracted using the QIAprep miniprep kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and electroporated into TIM2263 (Δ*yihT27*::kan). Strains with the vector (pWSK29) only were prepared similarly to controls.

Culture conditions and reporter assays.

All strains were maintained as frozen glycerol stocks, and were subcultured into LB with appropriate antibiotics (kanamycin at 50 μg/ml, ampicillin at 200 μg/ml, and tetracycline at 10 μg/ml) prior to the experiments. For plate assays, bacteria were seeded into soft LB or M9 agar (0.3% agar) with or without X-gal (40 μg/ml), as indicated in the text.

For the RIVET assays in tomato, *Salmonella* cultures were grown at 37°C overnight in LB supplemented with tetracycline. Bacterial cultures were then pelleted, washed three times in an equal volume of sterile phosphate-buffered saline (PBS), and diluted to 10^8 CFU/ml. Approximately 10^5 CFU (in 3 μl of water) were inoculated onto superficial 1-mm-deep wounds on surfaces of unwaxed fruit. At least three technical (individual infections) and two biological (tomato fruit from different plants) replications were carried out for each experiment. Unless otherwise stated, infected tomato fruit were incubated at 22°C in vented chambers. All RIVET assays were carried out for a week. To harvest samples, 15-by-0.5-mm cores were removed from fruit and homogenized in PBS, and aliquots were then plated onto xylose-lysine deoxycholate (XLD) agar (Oxoid, Hampshire, U.K.) with appropriate antibiotics. Individual colonies were then patched on LB agar with tetracycline to detect constructs in which TnpR recombinase was active.

Plant materials.

All assays were initially conducted in unwaxed red ripe Campari tomato fruit purchased at local supermarkets. Follow-up experiments, as indicated in text, were carried out with mature green or fully ripe tomato fruit of 'Amish Salad', 'Bonny Bes't, 'Celebrity', 'Red Calabash', 'Sebring', 'Solar Fire', 'Mariana', 'Bloody Butcher', 'Kumato', and 'Snow White'. Note that, at maturity (corresponding to the United States Department of Agriculture stages 5 and 6), fruit of 'Amish Salad', 'Bonny Best', 'Celebrity', 'Red Calabash', 'Sebring', 'Solar Fire', 'Mariana', and 'Bloody Butcher' turn red; 'Kumato' are brown; and 'Snow White' are ivory. Other than these pigmentation differences, 'Kumato' and 'Snow White' ripen normally. The brown color of ripe tomato fruit of 'Kumato' is due to an incomplete turnover of chlorophyll in mature tomato fruit. Seed was purchased from commercial suppliers. Tomato plants were grown in the field (two locations and seasons: Citra, FL in fall 2010 [conventional] and Archer, FL in spring 2012 [transitional organic]) or in the roof-top greenhouse (during the breaks between production seasons). For each variety, field and greenhouse-grown tomato fruit were sampled and the combined data are presented.

'Ailsa Craig' and lines nearly isogenic for the *rin* and *Nr* mutations (Vrebalov et al. 2002; Yen et al. 1995) were grown in the roof-top greenhouse in spring and summer 2012. To track developmental stages of the fruit, each developing fruit was tagged when it first reached exactly 1 cm in diameter, equal to 7 dpa (Alba et al. 2005). In the greenhouse, plants were grown from seed in Miracle-Gro potting soil and fertilized biweekly with Miracle-Gro tomato plant food (18-21-21) (Marysville, OH, U.S.A.).

For all samplings, fruit were harvested individually from the field- and greenhouse-grown plants, ensuring that ripe and unripe fruit were from the same plants to reduce any variability associated with differences in the associated endo- or epimicrobiota. All tomato fruit were inoculated with *Salmonella* spp. within 3 to 4 h of harvest.

For genotyping experiments, plants were grown in the greenhouse from seed to the approximately four- to six-true-leaf stage. DNA was extracted with a PowerPlant DNA isolation kit (MoBio, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. Genotyping for *Rin/rin* alleles was conducted by PCR using primers ATACGATAATGTACAACCCGAAAATG and TCAACTTGAACACACATAAAAAGGAA, yielding a 330-bp fragment diagnostic of the wild-type *Rin* allele, and primers CTTTCAAACATCATGGCATTGTGGTG and ATAT CATTGGCGGAACTTGACGTGAG, yielding a 765-bp fragment diagnostic for the mutant *rin* allele.

Fitness of the *Salmonella* mutants.

To calculate a competitive index, wild-type *S. enterica* sv. Typhimurium 14028 and isogenic mutants were seeded at 10^5 CFU/infection, roughly at a 1:1 ratio into tomato. In parallel, *S. enterica* sv. Typhimurium 14028 and its isogenic kanamycin-resistant strain CEC1000 were similarly inoculated onto eight tomato fruit, three wounds per fruit. All samples were incubated for a week at 22°C in vented chambers (with the exception of the ethylene add-back experiments; discussed below). Upon completion of the incubation, tomato fruit were inspected visually and those with signs of spoilage were discarded. At harvest, tomato fruit were macerated in a stomacher with an equal volume of PBS, then dilution plated onto XLD plates, which were incubated at 42°C. The relative ratios of the strains in the inocula and in the recovered samples were calculated by dilution plating and patching on antibiotic containing media. Competitive indices were calculated for each treatment using the formula $(M_{out}/WT_{out})/(M_{in}/WT_{in})$, where M is the proportion of mutant cells and WT is the proportion of the wild-type cells in the inocula (in) or in the recovered samples (out). Log-transformed values of competitive index are presented. The statistical and biological significance of each competitive index was established by comparing log values of the competitive indices of each pair to the log of competitive index similarly calculated for ATCC 14028 versus CEC1000, using the analysis of variance test ($P < 0.05$). It is important to recognize that, because of the way the fitness of the mutant is tested within the co-infection with the wild type, an increase in the fitness of the mutant could also be a consequence of the reduced fitness of the wild type under some conditions.

Extraction of hydrophobic components from tomato fruit.

Mature green (34 dpa) or red ripe (46 dpa) tomato fruit of 'Ailsa Craig' were blended and mixed with two volumes of chloroform. The organic phase was filtered (Whatman filter paper), rotary evaporated, and freeze dried overnight. For bioassays, 5 mg of lyophilized compounds were resuspended in 40 μl of dimethyl sulfoxide (DMSO). Controls containing only DMSO and distilled water were also tested. A volume of the extract corresponding to approximately 0.4 tomato "equivalents" was added to the LB 0.3% agar plates for the bioassays.

Ethylene add-back experiments.

Tagged, developmentally synchronized tomato fruit ('Ailsa Craig' wild type and *rin/rin*) were harvested at 34 and 46 dpa.

Tomato fruit were inoculated with an equal mix of TIM2263 (Δ*yihT*27::kan) and serovar Typhimurium 14028 exactly as above for fitness tests. Infected tomato fruit were placed inside a 40-by-40-by-20-cm lidded, air-tight aquarium, into which 0.39 ml of 100% ethylene was injected with a syringe for a treatment concentration of 12 ppm. Tomato fruit were incubated for 1 week and ethylene injections were repeated every 48 h, following a brief (approximately 10 min) venting to reduce accumulation of CO_2. Upon completion of the experiment, the 'Ailsa Craig' tomato fruit turned red while the *rin/rin* remained green. Tomato fruit were harvested and the competitive index was calculated as described above.

ACKNOWLEDGMENTS

This research was supported by the United States Department of Agriculture (USDA)-NRI AFRI grant 2011-67017-30127 (to M. Teplitski and J. J. Giovannoni). Construction of the *phoN* mutant was funded as a part of the project supported by the Center for Produce Safety, University of California-Davis (agreement number 201300842-01) and the California Department of Food and Agriculture's Specialty Crop Block Grant Program (SCB12064). C. E. Cox gratefully acknowledges USDA NIFA postdoctoral fellowship CRIS 0229689. We thank M. Moraes for assistance with designing primers to construct CEC1000.

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