Transgenic Expression of *EFR* and *Bs2* Genes for Field Management of Bacterial Wilt and Bacterial Spot of Tomato

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ABSTRACT

Field trials were conducted at two locations in Florida to evaluate transgenic tomato expressing the *ELONGATION FACTOR TU RECEPTOR* (*EFR*) gene from *Arabidopsis thaliana*, the *Bs2* gene from pepper, or both *Bs2* and *EFR* (*Bs2/EFR*) for managing bacterial wilt caused by *Ralstonia solanacearum* and bacterial spot caused by *Xanthomonas perforans*. Expression of *EFR* or *Bs2/EFR* in the susceptible genotype Fla. 8000 significantly reduced bacterial wilt incidence (50 to 100%) and increased total yield (57 to 114%) relative to lines expressing only *Bs2* or the nontransformed Fla. 8000 control, although the marketable yield was not significantly affected. Following harvest, surviving symptomatic and non-symptomatic plants were assessed for colonization by *R. solanacearum*. There were no significant differences in the population at the lower stem.

Interestingly, in the middle stem, no bacteria could be recovered from *EFR* or *Bs2/EFR* lines but viable bacterial populations were recovered from *Bs2* and nontransformed control lines at 10^2 to 10^5 CFU/g of stem tissue. In growth-chamber experiments, the *EFR* transgenic tomato lines were found to be effective against seven different *R. solanacearum* strains isolated from the southeastern United States, indicating utility across the southeastern United States. In all of the bacterial spot trials, *EFR* and *Bs2/EFR* lines had significantly reduced disease severity (22 to 98%) compared with the Fla. 8000 control. The marketable and total yield of *Bs2/EFR* were significantly higher (43 to 170%) than Fla. 8000 control in three of four field trials. These results demonstrate for the first time the potential of using the *EFR* gene for field management of bacterial wilt and bacterial spot diseases of tomato.

Tomato (*Solanum lycopersicum* Mill.) is an important vegetable crop in the United States and worldwide. The United States produced >1.2 million metric tons of fresh-market tomato in 2015, harvested from >38,000 ha of land with a farm gate value of \$1.2 billion (USDA-NASS 2017). However, bacterial wilt and bacterial spot can severely affect fresh-market tomato production. Bacterial wilt is caused by the soilborne bacterium *Ralstonia solanacearum* (Smith 1896) Yabuuchi et al. 1996 emend. Safni et al. 2014. *R. solanacearum* is a vascular pathogen that enters the plants through the roots and multiplies rapidly in the xylem vessels, producing vascular discoloration and wilting symptoms (Vasse et al. 1995). *R. solanacearum* has a host range of 450 plant species in 54 different families and thrives in hot and humid climates (Hayward 1991). It can survive in contaminated soil (van Elsas et al. 2000) and irrigation water (van Elsas et al. 2001) for many years, making it a difficult disease to manage. The predominant strain of *R. solanacearum* in the southeastern United States is phylotype II,

sequevar 7 (race 1, biovar 1). The disease can cause yield losses ranging from 80 to 100% when environmental conditions are favorable for disease development (Hong et al. 2012). Bacterial spot is caused by at least four distinct *Xanthomonas* spp.: *X. euvesicatoria* (race T1), *X. vesicatoria* (race T2), *X. perforans* (races T3 and T4), and *X. gardneri* (race T2) (Jones et al. 2004; Stall et al. 2009). Bacterial spot is a foliar disease that affects all aerial parts of the plant, resulting in leaf spots, defoliation, and lesions on fruit (Jones et al. 2014). The disease is estimated to cause yield losses of up to 50% when warm, humid conditions favor disease development (Horvath et al. 2012). Bacterial spot is a major problem in the southeastern United States, particularly affecting Florida, the leading state in production of fresh-market tomato. Bacterial wilt and bacterial spot have been reported in most of the tomato-producing regions of the world but currently available management strategies have limited success in effectively reducing disease incidence or severity (Nion and Toyota 2015; Potnis et al. 2015).

With regard to bacterial wilt, high costs associated with producing grafted plants and limited efficacy of chemical controls (Kunwar et al. 2015, 2017) warrant the development of effective, affordable, and environmentally friendly alternatives for disease control. Currently, there are no commercial varieties available in the United States that provide adequate resistance to bacterial wilt without compromising yield. Breeding for resistance to bacterial wilt is also difficult due to the presence of several pathogen races, lack of stable sources of resistance, complex inheritance, and the environmental effect on resistance (Scott et al. 2003b). Moreover, the resistance to bacterial wilt is linked with small fruit size and the

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transfer of resistance into market-preferred large-fruited backgrounds has proved challenging (Scott et al. 2003b).

Field management of bacterial spot has traditionally relied upon copper-based bactericides mixed with mancozeb or maneb (ethylene-bis-dithiocarbamate fungicides) but these bactericides are inadequate to provide full-season control because many of the xanthomonads associated with tomato in Florida are tolerant to copper (Horvath et al. 2012; Jones and Jones 1985; Obradovic et al. 2005). Applications of plant defense inducer acibenzolar S-methyl (ASM) (Actigard 50W; Syngenta Crop Protection, Greensboro, NC) have been reported to effectively reduce bacterial spot severity in the field (Obradovic et al. 2005); however, full-season application of ASM is not recommended due to its negative effect on yield (Graham and Myers 2013; Louws et al. 2001; Romero et al. 2001). Metallic-based nanomaterials are being evaluated as potential alternatives to copper-based bactericides (Ocoy et al. 2013; Paret et al. 2013, Strayer et al. 2016; Strayer-Scherer et al. 2018) but these nanomaterials are currently not available commercially and the long-term effect on human and environmental health is not known. Conventional breeding efforts have utilized both major resistance (*R*) genes and quantitative resistance loci for bacterial spot management in tomato (Scott et al. 2003a; Scott and Jones 1989). Although high levels of resistance were achieved using *R* genes, they rely on recognition of single pathogen effectors (Scott and Jones 1989). Due to the frequent mutations occurring in effectors, *R*-gene-based resistance may break down rapidly in the field, thereby rendering them ineffective even prior to commercial deployment. Ongoing efforts focus on selection for quantitative trait loci to obtain partial resistance. In contrast, transgenic resistance conferred by *Bs2*, an *R* gene from pepper (*Capsicum annuum*), provided effective field resistance to the predominant strain (race 4) of *Xanthomonas* spp. present in Florida and resulted in significant improvement to yield (Horvath et al. 2012). *Bs2* belongs to the nucleotide-binding site leucine-rich repeat family of innate immune receptors (Tai et al. 1999) and produces a hypersensitive response when it recognizes its cognate AvrBs2 protein, a highly conserved effector among xanthomonads (Kearney and Staskawicz 1990). The durability of *R* genes depends on the stability of the avirulence gene in the pathogen (Kearney and Staskawicz 1990), which has proved to be a limitation in deploying an *R* gene for bacterial spot management. Development of more durable and broad-spectrum management strategies against bacterial wilt and bacterial spot, while also improving tomato yield and quality, is crucial for the tomato industry in Florida and worldwide. Recently, a novel strategy was used to provide broad-spectrum bacterial resistance in tomato (*S. lycopersicum* 'Moneymaker') and tobacco (*Nicotiana benthamiana*), both members of Solanaceae family, against wide genera of bacterial pathogens (Lacombe et al. 2010). The interfamily transfer of a pattern recognition receptor (*PRR*) gene called *ELONGATION FACTOR TU RECEPTOR (EFR)* from *Arabidopsis thaliana* (Brassicaceae family) conferred effective resistance in tomato against *R. solanacearum* (GMI1000; race 1 biovar 3, phylotype I) and *X. perforans* (T4-4B) and in tobacco against *Agrobacterium tumefaciens* (A281), *Pseudomonas syringae* pv. *syringae* (B728a), and *P. syringae* pv. *tabaci* (11528) under greenhouse conditions (Lacombe et al. 2010). Recently, transgenic expression of *AtEFR*, was also demonstrated to enhance resistance in potato (*S. tuberosum*) against *R. solanacearum* (race 3, biovar 2A/ phylotype IIB, sequevar 1) (Boschi et al. 2017), as well as improved resistance in wheat (*Triticum aestivum* L.) against *P. syringae* pv. *oryzae*, the causal agent of halo blight of cereals (Schoonbeek et al. 2015) in greenhouse experiments. Transgenic expression of *AtEFR* has also been demonstrated to enhance defense response in rice (*Oryza sativa*) (Lu et al. 2015; Schwessinger et al. 2015). The *PRR* such as *EFR* are important components of pathogen-associated molecular pattern (PAMP)-triggered immunity and play an important role in plant defense by recognizing conserved PAMP in microbes (Boutrot and Zipfel 2017). It is important to test the

durability and sustainability of these *EFR* transgenic tomato lines in the field where complex cultural and environmental conditions exist. It is also important to evaluate the transgenic line against a range of tropical and subtropical strains of the pathogens to explore how broadly *EFR* may confer resistance to the diverse strains of the pathogen. The main objectives of this study were to (i) evaluate the effect of *EFR* and *Bs2* plus *EFR* (*Bs2/EFR*) genes on field-level management of bacterial spot and bacterial wilt disease and tomato yield, (ii) elucidate the movement of *R. solanacearum* from soil to root to crown and upward in the *EFR* and *Bs2/EFR* transgenic tomato lines by assessing the bacterial populations in the infected plants in the field, and (iii) evaluate the transgenic *EFR* tomato line against various strains of *R. solanacearum* in relevance to its potential application in bacterial wilt management in the southeastern United States.

MATERIALS AND METHODS

Bacterial culture and inoculum preparation. For bacterial wilt trials, a phylotype II sequevar 7 strain of *R. solanacearum* (Rs5; race 1, biovar 1) (Hong et al. 2012) was streaked on casamino acid-peptone-glucose agar (1 g of casamino acid, 10 g of peptone, 5 g of glucose, and 17 g of agar in 1 liter of deionized water) and incubated at 28°C (Kelman 1954). After 48 h, colonies of *R. solanacearum* were transferred to nutrient broth, placed on a shaker, and shaken at 200 rpm at 28°C. After 24 h, the bacterial suspension in nutrient broth was adjusted to an optical density at 600 nm (OD_{600}) = 0.1, which corresponds to 10^8 CFU/ml using sterile deionized water and diluted to 10^7 CFU/ml, which was used for inoculation in the growth-chamber experiments. For field inoculations, the bacterial suspension was further diluted to 10^5 CFU/ml using field irrigation water.

For bacterial spot trials in Balm, FL, *X. perforans* (race T4; strain mixture of GEV904, GEV917, GEV1001, and GEV1063) was used. The bacterial strains were grown on nutrient agar medium (BBL, Becton Dickinson and Co., Cockeysville, MD) and incubated at 28°C for 24 h. The bacterial cells were removed from the plates and suspended in a 10 mM $MgSO_4 \cdot 7H_2O$ solution, and the suspension was adjusted to OD_{600} = 0.3, which corresponds to 10^8 CFU/ml. The bacterial suspension for each strain was then diluted to 10^6 CFU/ml and mixed in equal volume and applied along with polyoxyethylene sorbitan monolaurate (Tween 20; 0.05% [vol/vol]) for field inoculation.

Plant lines. Field trials were conducted under United States Department of Agriculture (USDA) notifications using Fla. 8000 and Fla. 8314 fresh-market backgrounds, both of which were developed by J. W. Scott at the University of Florida. Fla. 8000 is a large-fruited parental line which has hypersensitive resistance to *X. perforans* race T3 derived from Hawaii 7981 (Wang et al. 2011) but which is susceptible to race T4. Fla. 8314 is a large-fruited hybrid derived from the cross between Fla. 8000 and Fla. 8111B, the latter of which is highly susceptible to all races of bacterial spot. Fla. 8314 is likewise susceptible to bacterial spot race T4, and both Fla. 8000 and Fla. 8314 are susceptible to bacterial wilt. The *Bs2* or *EFR* transgenes were backcrossed into the Fla. 8000 background to generate near-isogenic transgenic lines. Fla. 8000-*EFR* was produced by crossing Fla. 8000 with the transgenic Moneymaker line expressing *EFR* under control of the constitutive 35S promoter (Lacombe et al. 2010) and then continuing the backcrossing to the sixth generation (BC₆). The single-copy insertion of *EFR* in the original Moneymaker background was determined using quantitative real-time polymerase chain reaction (PCR) by Lacombe et al. (2010), which was used as the donor of *EFR* in this study. Homozygous BC₄ lines were used in bacterial wilt trials and homozygous and hemizygous BC₅ and BC₆ lines were used in bacterial spot trials. Fla. 8000-*Bs2* was produced by crossing Fla. 8000 with the transgenic VF36 line expressing *Bs2* under control of the 35S promoter (Horvath et al. 2012), then continuing the

backcrossing to the BC₄ generation. Fla. 8000-*Bs2/EFR* was produced similarly as Fla. 8000-*EFR*, except that the Fla. 8000-*Bs2* line was used as the recurrent parent; backcrossing continued to the BC₆ generation. Fla. 8314-*Bs2* was produced by crossing Fla. 8000-*Bs2* with Fla. 8111B and Fla. 8314-*Bs2/EFR* was produced by crossing Fla. 8111B-*Bs2* with Fla. 8000-*EFR*.

In field trials conducted in Quincy, FL, Fla. 8000 lines were compared with the three Fla. 8000 near-isogenic transgenic lines for their ability to reduce bacterial wilt symptoms and improve yield. In field trials in Balm, FL, Fla. 8000 and Fla. 8314 were compared with each of the Fla. 8000 near-isogenic transgenic lines and with Fla. 8314-*Bs2* and Fla. 8314-*Bs2/EFR*. Trials in Balm also included the large-fruited commercial hybrids Florida 47, Sanibel, and Florida 91 (Seminis Vegetable Seeds, St. Louis) as commercial controls.

Field experiments. Two field trials were conducted at the University of Florida, North Florida Research and Education Center (NFREC) in Quincy during the fall seasons of 2015 and 2016 to evaluate the *EFR* and *Bs2/EFR* transgenic tomato lines against bacterial wilt disease of tomato. The soil type in Quincy was Norfolk sandy loam (Thermic Typic Kandiudults), pH 6.3, and had been used the previous 5 years for conducting bacterial wilt trials of tomato in the fall and left fallow during the rest of the year. Prior to that, the plot area had been used to grow tomato in the fall or cucurbits in summer.

For bacterial spot experiments, four field trials were also conducted at the University of Florida, Gulf Coast Research and Education Center (GCREC) in Balm during fall 2015, spring 2016, fall 2016, and spring 2017 to evaluate the *EFR* and *Bs2/EFR* transgenic tomato lines against bacterial spot of tomato. Soil type in Balm was Myakka fine sand (sandy, siliceous, hyperthermic Oxyaquic Alorthod); the trial site was used for conducting all of the bacterial spot experiments.

All the field trials in Quincy and in Balm were conducted using a randomized complete block design and under the notification process of the United States Department of Agriculture. The bacterial wilt trials consisted of four plots, with 14 and 12 plants per plot in the 2015 and 2016 trials, respectively. The number of plants per plot varied in each year and was based on the number of seed that were available in the particular year. Experimental plots for both bacterial wilt trials consisted of fumigated raised beds having dimensions of 20.3 cm tall by 76.2 cm wide, spaced 51 cm between plants, and covered with white polyethylene mulch. Fumigation was conducted with chloropicrin (50%) and methyl bromide (50%) applied at a rate of active ingredients at 280 kg/ha. Seed of each line were grown in polystyrene flats containing peat vermiculite mixture (Metro Mix; Sun Gro Horticulture Canada Ltd., Agawam, MA) in a greenhouse for 5 to 6 weeks before they were transplanted to the field. Field inoculations in fall 2015 were conducted on 6 August (7 days before transplantation) and in fall 2016 on 15 August (3 days before transplantation) by pouring 50 ml of inoculum at 10⁵ CFU/ml per planting hole. Bacterial wilt incidence was recorded at weekly intervals until harvest as the proportion of the diseased plants (i.e., the number of plants per plot showing disease symptoms divided by the total number of plants in that plot), expressed as percentage. Bacterial wilt severity data were calculated using a 0-to-4 bacterial wilt rating scale, where 0 = no leaf area wilted, 1 = 1 to 25% of leaves wilted, 2 = 26 to 50% of leaves wilted, 3 = 51 to 75% of leaves wilted, and 4 = 76 to 100% of leaves wilted (Jakobs et al. 2011). The percentage ratings were used for statistical analysis. Fruit were harvested from each plot twice (on 18 November and 1 December 2015 and on 9 and 28 November 2016). In the bacterial spot trials, each treatment in fall 2015 and fall 2016 consisted of three plots with 10 plants per plot, and in spring 2016 and 2017 consisted of four plots with 8 and 10 plants per plot, respectively. Bed dimensions were 20.3 cm high and 81 cm wide, spaced 46 cm between plants. Fumigation was conducted with a 60:30 combination of chloropicrin and 1, 3-dichloropropene (Pic-Clor 60 EC at 336.26 kg/ha; Soil Chemical Corporation, Hollister, CA) and covered with black or white plastic mulch for spring or fall trials,

respectively. The plants were transplanted to the field on 21 August (fall 2015), 25 March (spring 2016), 23 August (fall 2016), and 24 March (spring 2017). Inoculations were done on 9 September (fall 2015), 26 April (spring 2016), 10 September (fall 2016), and 13 April (spring 2017) by spraying 10⁶ CFU/ml of the bacterial suspension on the foliage of all plants with a backpack sprayer (Model 425; Solo Incorporated, Newport News, VA). Bacterial spot severity was rated from 4 to 8 weeks after inoculation using the Horsfall-Barratt scale (Horsfall and Barratt 1945) and converted to midpoint percentages for statistical analysis. Fruit were harvested three to four times for each trial. The bacterial wilt and bacterial spot trial plots were maintained throughout the season using standard fertility, irrigation, and crop protection practices recommended for commercial tomato production in Florida (Dittmar et al. 2015) and with no applications of copper, plant defense inducers, or other bactericides. The fruit in the Quincy and Balm trials were graded for fruit size and marketability according to USDA specifications (USDA-AMS 1991). The total yield was calculated by adding yield from marketable (≥ 5.71 cm) and small (< 5.71 cm) fruit.

The weather conditions for each of the bacterial wilt and bacterial spot trials were recorded. The weather data were obtained from the University of Florida, Florida Automated Weather Network website (<https://fawn.ifas.ufl.edu/>). Eight-year monthly averages (henceforth known as typical monthly average) of temperatures, total rainfall, soil temperature, and relative humidity were calculated by averaging values for each month over an 8-year period from April 2010 through August 2017. Monthly temperature, total rainfall, soil temperature, and relative humidity data were also recorded for each month of a specific year in which plants were in the field and expressed as a percentage of the 8-year monthly average (Supplementary Tables S1 and S2). The impact of expressing *EFR* and *Bs2/EFR* genes in the Fla. 8000 background on marketable yield under different environmental conditions and disease pressure was determined by dividing the average marketable yield of *EFR* or *Bs2/EFR* plants by the average marketable yield of Fla. 8000 plants, using the method used for calculating *Bs2* impact factor by Horvath et al. (2012). The impact factors are based on numeric values and do not represent the statistically significant values.

Assessment of the *R. solanacearum* colonization in the surviving symptomatic and asymptomatic plants in the field. Following harvest, the middle and lower stems of all of the surviving symptomatic and asymptomatic plants in the bacterial wilt trials were assessed for the recovery of *R. solanacearum* colonies. Lower stem samples were taken from 2 cm above the soil line and middle stem samples were taken at half of the plant height. Stem sections (1 g = approximately 10 cm) were weighed, surface sterilized with 10% bleach, and rinsed twice with sterile deionized water. Tissues were then chopped into 1-cm pieces with a sterile blade and placed inside a Ziploc bag filled with 100 ml of sterile deionized water for 20 min with constant shaking. A series of 10-fold dilutions (10⁻¹ to 10⁻⁶) of the initial suspension were made and 100 μ l each of 10⁻¹, 10⁻², 10⁻⁴, and 10⁻⁶ dilutions were plated on a modified semi-selective medium from South Africa (SMSA) media using the spread plate technique (Elphinstone et al. 1996). After 48 h, the plates were assessed for the presence of *R. solanacearum* colonies. The percentages of lower and middle stems with recoverable amounts of *R. solanacearum* in SMSA media were determined. Furthermore, *R. solanacearum* colonies observed in the SMSA plates were counted and *R. solanacearum* population was determined using the formula bacterial population = number of colonies $\times$ dilution factor $\times$ 10 and expressed as log CFU per gram of tissue. Only the stem samples from which colonies of *R. solanacearum* were recovered in SMSA media were considered to calculate mean population per plot and for statistical analysis.

Evaluation of transgenic *EFR* tomato lines against *R. solanacearum* strains collected from the southeastern United States. Two independent growth-chamber experiments were conducted to evaluate transgenic *EFR* lines against seven

different strains of *R. solanacearum* collected from Florida, Georgia, North Carolina, and Alabama (Hong et al. 2012). All strains collected were race 1 and biovar 1. Detailed information about the strains is provided in Table 1. Nontransformed Fla. 8000 plants were used as the susceptible control. The plants were inoculated at the three- to four-leaf stage by pouring 50 ml of bacterial suspension (at 10^7 CFU/ml) onto the soil at the base of each plant and maintaining plants inside the growth chamber with a temperature of 20 to 24°C at night and 32 to 38°C during daytime with a 16 h-photoperiod throughout the experiment. The plants were watered twice daily (approximately 50 ml every afternoon and evening) throughout the experiment. The plants were scored daily for bacterial wilt severity using the 0-to-4 scale described above, and mean percent disease severity was calculated. There were five plants per treatment per experiment. The noninoculated controls of Fla. 8000 and Fla. 8000-*EFR* were also included in both experimental repeats.

Statistical analyses. All disease ratings and yield data were analyzed using one-way analysis of variance in R (R Core Team 2016). Mean separation was conducted using Fisher's least significant difference at $P = 0.05$. The percentage of stems from which *R. solanacearum* colonies were recovered in semiselective media was analyzed using Kruskal-Wallis test followed by Wilcoxon Each Pair test in JMP software of SAS (version 12; SAS Institute Inc., Cary, NC). The bacterial population data were analyzed as a split-plot design using JMP software of SAS and data for each year were analyzed separately. The lower and middle stems were used as the whole-plot factor and the treatment as the subplot factor. Block was treated as a random factor. Pairwise comparison of means was conducted using the Tukey's honestly significant difference multiple comparison procedure at $P = 0.05$. For the growth-chamber experiments, the disease severity data of each strain were analyzed separately using the Mann-Whitney U test in IBM SPSS Statistics (IBM Corp. 2013). Because no wilting was observed in any of the noninoculated Fla. 8000 and Fla 8000-*EFR* controls, they were not included in the statistical analysis.

TABLE 1. List of *Ralstonia solanacearum* isolates from tomato in the southeastern United States used in the growth-chamber experiments against *ELONGATION FACTOR TU RECEPTOR* transgenic tomato lines^z

Strain number	Strain ID	Location	Phylotype	Sequevar
1	Rs5	North Florida	II	7
2	Rso 81-5	Georgia	II	Unknown
3	AW1	Alabama	II	7
4	P507	Central Florida	II	4
5	RS136	Florida	II	Unknown
6	RS137	Florida	II	Unknown
7	K136	North Carolina	II	7

^z Strain information was obtained from Hong et al. (2012).

RESULTS

The impact of *EFR* and *Bs2/EFR* transgenes in tomato on bacterial wilt disease and fruit yield. Bacterial wilt incidence in the *EFR* and *Bs2/EFR* transgenic lines was significantly reduced from 70 to 82% to 16 to 35% in 2015 and from 23 to 33% to 0% in 2016 relative to the *Bs2* line and nontransformed control (Table 2; Supplementary Fig. S1). Bacterial wilt severity was also significantly reduced in the *EFR* and *Bs2/EFR* transgenic lines from 58 to 71% to 13 to 30% in 2015 and from 19 to 28% to 0% in 2016 relative to the *Bs2* line and nontransformed control (Table 2). Numbers of marketable fruit from *EFR* or *Bs2/EFR* lines were numerically higher but statistically similar to those from *Bs2* lines and nontransformed controls. However, the total yield of *EFR* or *Bs2/EFR* lines was significantly increased from approximately 25,000 to 26,000 kg/ha to approximately 41,000 to 52,000 kg/ha compared with *Bs2* and nontransformed lines in 2015 and from approximately 12,000 to 15,000 kg/ha to approximately 23,000 to 26,000 kg/ha in 2016 (Table 3).

The overall monthly temperatures, soil temperature, and relative humidity were generally within 5% of 8-year averages during early seasons (August to September) of both years (Table 4). However, during the late season (i.e., in November, when the final disease data were taken and reported in this study), the values were above 5% of 8-year averages in 2015 whereas they remained within 5% of 8-year averages in 2016. Corresponding to this, high disease pressure was observed in 2015 (82% maximum disease incidence in the Fla. 8000 control) and relatively low disease pressure was observed in 2016 (23% maximum disease in the Fla. 8000 control) (Table 4).

The resistance of the *EFR* and *EFR/Bs2* transgenic lines against *R. solanacearum* is partially due to restricted bacterial colonization and movement within the host. The transgenic line expressing the *EFR* gene alone had a significantly reduced percentage of lower and middle stems with recoverable amounts of *R. solanacearum* relative to the *Bs2* line (P values for middle and lower stems were 0.0211 and 0.0304, respectively, for both years) or the Fla. 8000 nontransformed control (P values for middle and lower stems were 0.0202 and 0.0294, respectively, for 2015 and 0.0211 and 0.0294, respectively, for 2016) (Table 5). The transgenic line expressing both *EFR* along with *Bs2* genes also had a significantly reduced percentage of lower and middle stems with recoverable amounts of *R. solanacearum* relative to the *Bs2* line (P values for middle and lower stems were 0.0211 and 0.0294, respectively for both years) or the Fla. 8000 nontransformed control (P values for middle and lower stems were 0.0202 and 0.0294, respectively, in 2015 and 0.0211 and 0.0294, respectively, in 2016) (Table 5). The population of *R. solanacearum* in the lower and middle stems was significantly dependent on the plant genotype (P values were 0.0002 in 2015 and <0.0001 in 2016), location of stem (P values were 0.0059 in 2015 and 0.0002 in 2016), and their

TABLE 2. Comparison of nontransgenic tomato lines and transgenic tomato genotypes expressing *ELONGATION FACTOR TU RECEPTOR* (*EFR*) and *Bs2/EFR* on bacterial wilt (BW) disease incidence and BW severity^x

Entry ^z	Fall 2015 ^y		Fall 2016 ^y	
	Incidence (%)	Severity (%)	Incidence (%)	Severity (%)
Fla. 8000	82 a	71 a	23 a	19 a
Fla. 8000- <i>Bs2</i> **	70 a	58 a	33 a	28 a
Fla. 8000- <i>EFR</i> ** (BC ₄)	16 b	13 b	0 b	0 b
Fla. 8000- <i>Bs2</i> **/ <i>EFR</i> ** (BC ₄)	35 b	30 b	0 b	0 b
Pr > F	<0.0001	<0.0001	0.0093	0.0082

^x Field trials were conducted during the fall seasons of 2015 and 2016 in Quincy, FL. Column means followed by the same letter do not differ significantly at $P = 0.05$ based on least significant difference.

^y Percentage BW incidence and BW severity were analyzed separately using one-way analysis of variance.

^z Each entry consisted of four replications with 14 plants per replication in the 2015 trial and 12 plants per replication in the 2016 trial. Asterisks (**) indicate homozygous transgenic and BC = backcross. The field was inoculated with 50 ml of *R. solanacearum* (race 1, biovar 1, phylotype II, sequevar 7) at 10^5 CFU/ml in each planting hole. Field trials were conducted using a randomized complete block design.

interaction (genotype–stem location) (P values were 0.0311 in 2015 and 0.0002 in 2016) (Fig. 1). The mean separation of the interaction showed no significant differences in the population at the lower stem (Fig. 1). Strikingly, in the middle stem, no bacteria could be recovered from the *EFR* line or *Bs2/EFR* line but viable bacterial populations were recovered from *Bs2* and nontransformed control lines at 10^2 to 10^5 CFU/g of tissue (Fig. 1).

EFR provides broad-spectrum resistance in tomato against several strains of R. solanacearum of the southeastern United States. For all of the *R. solanacearum* strains tested in the study, disease severity was significantly reduced in the *EFR* line compared with the nontransformed Fla. 8000 control for both experimental repeats (Fig. 2). Wilting symptoms were not observed in any of the Fla. 8000 and Fla. 8000-*EFR* noninoculated controls (data not shown).

Effect of EFR and Bs2/EFR genes on bacterial spot severity and fruit yield in tomato. In all of the bacterial spot field trials, Fla. 8000

carrying *EFR*, alone or in combination with *Bs2*, significantly reduced bacterial spot severity relative to all of the commercial lines and the nontransformed Fla. 8000 control (Table 6; Supplementary Fig. S2). The *EFR* gene alone was less effective in reducing disease as compared with the *Bs2/EFR* combination or *Bs2* gene alone (Table 6). The level of disease reduction achieved in the *EFR* line or *Bs2/EFR* line, relative to the nontransformed control, was not affected by whether the *EFR* transgene was in homozygous or hemizygous condition (Table 6). Consistent with previous results (Horvath et al. 2012), the *Bs2* gene expressed in Fla. 8000 and Fla. 8314 in homozygous and hemizygous condition, respectively, also significantly reduced bacterial spot severity relative to all of the commercial lines and the nontransformed Fla. 8000 and Fla. 8314 controls (Table 6).

In the fall 2015 trial, Fla. 8000-*Bs2/EFR* (BC₅) had significantly higher marketable and total yields relative to all of the commercial lines and nontransformed Fla. 8000 control (Table 7). The total yield of the Fla. 8000-*Bs2/EFR* line was statistically similar to Fla. 8000-*Bs2* although the marketable yield was significantly less than Fla. 8000-*Bs2* (Table 7). As demonstrated previously (Horvath et al. 2012), the *Bs2* gene was found to have a positive effect on tomato yield. The marketable and total yield of Fla. 8000-*Bs2* lines (homozygous for *Bs2*) was significantly higher than the nontransformed Fla. 8000 control. The marketable and total yield of Fla. 8314-*Bs2* (heterozygous for *Bs2*) was also significantly higher than the nontransformed Fla. 8314 control (Table 7).

In the spring 2016 trial, the total and marketable yield of Fla. 8000-*Bs2/EFR* (BC₅ and BC₆) was numerically higher (statistically not significant) than the commercial lines or nontransformed Fla. 8000 control and statistically equivalent to line Fla. 8000-*Bs2* (Table 7). Similar to the results obtained in fall 2015, the Fla. 8000-*Bs2* line, homozygous for *Bs2*, produced significantly higher marketable and total yield compared with the nontransformed Fla. 8000 control. Fla. 8314-*Bs2*, hemizygous for *Bs2*, did not provide statistically higher marketable and total yields relative to the nontransformed Fla. 8314 control (Table 7).

In the fall 2016 trial, *EFR* in combination with *Bs2* also had positive impact on yield relative to the commercial lines and nontransformed control similar to what was obtained in the fall 2015 trial. The total and marketable yields of Fla. 8000-*Bs2/EFR* were significantly higher than commercial lines and the nontransformed Fla. 8000 control. Also, the total and marketable yields of Fla. 8000-*Bs2/EFR*, regardless of the homozygosity of *EFR* gene, were statistically comparable with line *Bs2* (Table 7). Fla. 8000-*Bs2*, homozygous for *Bs2*, produced significantly greater marketable and total yield than the nontransformed Fla. 8000 control (Table 7). Similarly, Fla. 8314-*Bs2*, heterozygous for *Bs2*, also produced significantly higher marketable and total yield than the nontransformed Fla. 8314 control (Table 7). *EFR* alone, in the hemizygous state, was also found to confer a positive impact on marketable yield relative to commercial lines and the nontransformed Fla. 8000 control. Expression of *EFR* in the hemizygous state, in the Fla. 8000 background, significantly increased marketable yield relative to commercial controls and the nontransformed Fla. 8000 control and was statistically equivalent to Fla. 8000-*Bs2* (Table 7). In contrast, homozygous *EFR* Fla. 8000 had marketable yield statistically equivalent to commercial lines and the nontransformed Fla. 8000 control and was significantly less than Fla. 8000-*Bs2* (Table 7).

In the spring 2017 trial, the *Bs2/EFR* plants in either the Fla. 8000 or Fla. 8314 parental backgrounds had significantly improved marketable and total yields relative to the respective nontransformed controls (Fla. 8000 and Fla. 8314) and commercial lines. This result was consistent with results obtained in fall 2015 and fall 2016. *Bs2* alone in the Fla. 8000 or Fla. 8314 parental backgrounds also significantly improved marketable and total yields relative to the respective nontransformed controls (Fla. 8000 and Fla. 8314) and commercial lines regardless of zygosity of the *Bs2* gene (Table 7). The marketable and total yields of Fla. 8000 and Fla. 8314 expressing both *Bs2* and *EFR* transgenes were statistically equivalent to Fla. 8000 and Fla.

TABLE 3. Comparison of tomato yield in nontransgenic tomato lines and transgenic tomato genotypes expressing *ELONGATION FACTOR TU RECEPTOR (EFR)* and *Bs2/EFR* in the field inoculated with *Ralstonia solanacearum*^x

Entry ^z	Yield (kg/ha) ^y			
	Marketable		Total	
	2015	2016	2015	2016
Fla. 8000	19,217 a	10,162 a	24,705 b	14,801 b
Fla. 8000- <i>Bs2</i> **	17,254 a	6,678 a	25,861 b	12,261 b
Fla. 8000- <i>EFR</i> ** (BC ₄)	27,901 a	12,338 a	52,201 a	26,264 a
Fla. 8000- <i>Bs2</i> **/ <i>EFR</i> ** (BC ₄)	25,753 a	11,348 a	40,653 a	23,275 a
$P > F$	0.1655	0.2922	0.0041	0.0033

^x Field trials were conducted during the fall seasons of 2015 and 2016 in Quincy, FL. Column means followed by the same letter do not differ significantly at $P = 0.05$ based on least significant difference.

^y Marketable yield was recorded at harvest and calculated based on United States Department of Agriculture specifications by adding marketable (≥ 5.71 cm) fruit. Total yield was calculated by adding yield from marketable and small (< 5.71 cm) fruit. Marketable and total yield were analyzed separately for each year using one-way analysis of variance.

^z Each entry consisted of four replications with 14 plants per replication in 2015 and 12 plants per replication in 2016. Asterisks (**) indicate homozygous transgenic and BC = backcross. The field was inoculated with 50 ml of *R. solanacearum* (race 1, biovar 1, phylotype II, sequevar 7) at 10^5 CFU/ml in each planting hole. Field trials were conducted using randomized complete block design.

TABLE 4. Summary of the bacterial wilt field trial conditions and impact factor of *ELONGATION FACTOR TU RECEPTOR (EFR)* and *Bs2/EFR*^s

	Bacterial wilt trial (Quincy)	
	Fall 2015	Fall 2016
Temperature ^t	Normal, hot late	Normal
Rainfall ^u	Uneven, high late	Uneven, low late
Soil temperature ^v	Normal, hot late	Normal
Relative humidity ^w	Normal, high late	Normal, low late
Disease pressure ^x	High	Low
Impact factor of <i>EFR</i> ^y	1.5	1.2
Impact factor of <i>Bs2/EFR</i> ^z	1.3	1.1

^s Bacterial wilt trials were conducted during fall seasons of 2015 and 2016 in Quincy, FL.

^t Normal: within 5% of typical averages.

^u Total rainfall relative to typical monthly totals.

^v Normal soil temperature is within 5% of typical averages.

^w Relative humidity is normal if the value is within 5% of typical averages.

^x Relative disease pressure based on bacterial wilt severity ratings. High: $> 70\%$ severity rating in Fla. 8000 and low: $\leq 20\%$ severity rating in Fla. 8000.

^y *EFR* impact factor was calculated by dividing the marketable yield for Fla. 8000-*EFR* plants with the marketable yield for Fla. 8000 plants.

^z *Bs2/EFR* impact factor was calculated by dividing the marketable yield for Fla. 8000-*Bs2/EFR* plants with the marketable yield for Fla. 8000 plants.

8314 expressing the *Bs2* transgene alone (Table 7). Hemizygous *EFR* plants in the Fla. 8000 background had significantly increased marketable yields relative to commercial controls and the non-transformed Fla. 8000 control and were statistically equivalent to Fla. 8000-*Bs2* (Table 7). Similarly, when *EFR* was expressed in homozygous condition in the Fla. 8000 background, the marketable yield remained statistically equivalent to commercial lines and the non-transformed Fla. 8000 control and was significantly less than Fla. 8000-*Bs2* (Table 7), similar to the results obtained in the fall 2016 trial.

In the bacterial spot trials, the overall, monthly temperatures and relative humidity throughout the trials remained within 5% of 8-year averages during the months when disease data were taken (i.e., March to June for spring trials and August to October for fall trials), whereas the total rainfall was unevenly distributed and

deviated from the typical averages. Also, the bacterial spot severity was relatively consistent across trials (ranging from 48 to 60% for Fla. 8000) except in spring 2016, when the severity was relatively low (19.2% for Fla. 8000) (Table 6). Despite the difference in the environmental conditions, *EFR* displayed a 1.1- to 1.8-fold enhancement of marketable yield and *Bs2*/*EFR* displayed a 1.1 to 2.5-fold enhancement of marketable yield relative to the non-transformed Fla. 8000 control (Table 8).

DISCUSSION

Improving plant resistance is the most sustainable, cost effective, and environmentally sound strategy to manage plant disease. However, breeding tomato for resistance to bacterial wilt and

TABLE 5. Percentage of stems with recoverable amounts of *Ralstonia solanacearum* in modified semi-selective medium from South Africa (SMSA) media^w

Year, entry ^z	Number of total surviving plants in field after harvesting			Number of stems plated on SMSA ^x		Number of stems with colonies present		Stems with colonies present (%) ^y	
	Sym.	Nonsym.	Total	L ¹	M ¹	L ²	M ²	L ³	M ³
2015									
Fla. 8000	18	9	27	27	22	23	14	85 a	64 a
Fla. 8000- <i>Bs2</i>	23	14	37	37	33	28	20	75 a	61 a
Fla. 8000- <i>EFR</i>	2	42	44	44	41	12	0	27 b	0 b
Fla. 8000- <i>Bs2</i> / <i>EFR</i>	10	27	37	37	35	5	0	13 b	0 b
2016									
Fla. 8000	9	37	46	46	42	18	19	39 A	45 A
Fla. 8000- <i>Bs2</i>	11	31	44	44	36	14	12	32 A	36 A
Fla. 8000- <i>EFR</i>	2	46	48	48	41	7	0	15 B	0 B
Fla. 8000- <i>Bs2</i> / <i>EFR</i>	0	48	48	48	47	13	0	27 B	0 B

^w Bacterial wilt trials were conducted in Fall season of 2015 and 2016 in Quincy, FL. Sym. = symptomatic, Nonsym. = nonsymptomatic, L = lower stem, and M = middle stem. Column means followed by the same letter do not differ significantly based on Wilcoxon Each Pair test.

^x Stems were sampled from all the surviving (symptomatic and nonsymptomatic) plants in the field following harvesting.

^y Calculated by dividing the number of stems with *R. solanacearum* colonies present by total stems plated on SMSA media and expressed as percentage; that is, $L^3 = (L^2/L^1) \times 100\%$ and $M^3 = (M^2/M^1) \times 100\%$. Data on the middle and lower stems within each year were analyzed separately using Kruskal-Wallis test followed by Wilcoxon Each Pair test.

^z All the transgenic lines were homozygous for the transgene.

Fig. 1. Comparing *Ralstonia solanacearum* population in the lower and middle stem tissue of the nontransgenic and transgenic tomato lines from which viable *R. solanacearum* colonies were recovered in modified semi-selective medium from South Africa (SMSA) media. Bars represent the means of four plots. All transgenic lines were homozygous for the transgenes *ELONGATION FACTOR TU RECEPTOR (EFR)*, *Bs2*, or both (*Bs2*/*EFR*). Because no viable colonies of *R. solanacearum* were recovered from the middle stem of *EFR* and *Bs2*/*EFR* lines, all of the values were increased by one unit before converting to log values. Data for each year were analyzed separately as a split-plot design using lower and middle stems as whole-plot and treatment as subplot factors. Block was used as random factor. Error bars represent standard error of mean. Bars with the same letter do not differ significantly at $P = 0.05$ based on Tukey's honestly significant difference test.

bacterial spot is difficult for several reasons. One difficulty is having to breed resistance to multiple bacterial species and races of pathogens and another is the lack of an easy method for screening plants for resistance to these diseases (Genin and Boucher. 2002; Jones et al. 1998; Monma and Sakata 1993; Opena et al. 1990; Wang et al. 1998; Wencai and Francis 2005). In the case of bacterial wilt, breeding for resistance in tomato plants is further complicated by complex quantitative inheritance and association between disease resistance and small fruit size (Opena et al. 1990; Wang et al. 1998), which has prevented the development of commercially preferred large-fruited tomato with adequate resistance to bacterial wilt. The use of a transgenic approach presents an alternative and efficient

means of transfer of disease resistance genes into commercially accepted varieties without compromising other horticultural traits. In this study, we demonstrated that the transgenic expression of *EFR*, a PRR from *Arabidopsis thaliana* (Brassicaceae) when expressed in tomato (Solanaceae), alone or in combination with the *Bs2* transgene from pepper, provided effective field level control of bacterial wilt and bacterial spot of tomato. To the best of our knowledge, this is the first study demonstrating improved resistance in tomato based on an interfamily transfer of a plant PRR gene providing disease resistance to multiple pathogens under field conditions. This study demonstrates that PRR transferred from one plant family (Brassicaceae) to another (Solanaceae) can provide

R. solanacearum strains collected from southeast U.S.

Fig. 2. Comparison of nontransgenic tomato line and transgenic tomato genotypes expressing the *ELONGATION FACTOR TU RECEPTOR (EFR)* gene on bacterial wilt severity caused by *Ralstonia solanacearum* strains collected from the southeastern United States. The *EFR* transgenic lines were homozygous for the transgenes. Two independent experiments were conducted, with five replications per treatment. Data for each strain were analyzed separately using the Mann-Whitney *U* test. Bars with an asterisk (*) represent statistically significant values. Error bars represent the standard error of mean.

TABLE 6. Comparison of nontransgenic tomato lines and transgenic tomato lines expressing *ELONGATION FACTOR TU RECEPTOR (EFR)* and *Bs2/EFR* on bacterial spot (BS) severity in the field trials conducted in Balm, FL during fall 2015, spring 2016, fall 2016, and spring 2017

Entry ^z	BS severity (%) ^y			
	Fall 2015	Spring 2016	Fall 2016	Spring 2017
FL47	47.7 ab	40.3 a	42.8 b	67.2 a
FL91	32.4 c	24.6 bc	42.5 b	56.3 a
Sanibel	40.3 bc	33.7 ab	57.0 a	56.3 a
Fla. 8000	48.3 ab	19.2 c	60.0 a	56.3 a
Fla. 8314	55.0 a	37.6 a	63.0 a	67.2 a
Fla. 8000- <i>Bs2</i> ** (BC ₄)	9.1 d	3.4 d	1.6 d	4.7 c
Fla. 8314- <i>Bs2</i> *	14.2 d	3.0 d	1.5 d	5.3 c
Fla. 8000- <i>EFR</i> ** (BC ₆)	Nt	Nt	33.1 c	37.5 b
Fla. 8000- <i>EFR</i> * (BC ₆)	Nt	Nt	30.1 c	43.8 b
Fla. 8000- <i>Bs2</i> **/ <i>EFR</i> * (BC ₅)	2.5 d	1.8 d	Nt	Nt
Fla. 8000- <i>Bs2</i> **/ <i>EFR</i> * (BC ₆)	Nt	2.3 d	1.5 d	3.5 c
Fla. 8000- <i>Bs2</i> **/ <i>EFR</i> ** (BC ₆)	Nt	Nt	1.6 d	4.7 c
<i>P</i> > <i>F</i>	<0.0001	<0.0001	<0.0001	<0.0001

^y BS severity was recorded using Horsfall-Barratt scale (Horsfall and Barratt 1945) and converted to midpoint percentages for statistical analysis. BS severity data were analyzed separately for each year using one-way analysis of variance at *P* = 0.05. Column means followed by the same letter do not differ significantly at *P* = 0.05 based on least significant difference.

^z In the bacterial spot trials, each treatment in fall 2015 and fall 2016 consisted of three replications with 10 plants per replication and in spring 2016 and 2017 consisted of four replications with 8 and 10 plants per replication, respectively. Asterisks ** = homozygous and * = hemizygous, Fla. 8314 = F1 control, BC = backcross, and Nt = not tested. Fields were inoculated by spraying *Xanthomonas perforans* at 10⁶ CFU/ml on the foliage of all plants with a backpack sprayer, applied along with polyoxyethylene sorbitan monolaurate (Tween 20; 0.05% vol/vol). Field trials were conducted using a randomized complete block design.

effective field-level resistance to multiple diseases in the field, and encourages identification of additional PRR for engineering broad spectrum disease resistance in plants against multiple pathogens under production conditions.

Growth-chamber experiments revealed *EFR* lines to be effective against seven strains of *R. solanacearum* from the southeastern United States (Fig. 2), demonstrating that the resistance is not strain specific and suggesting the potential for use of this gene across the southeastern United States. Further evaluation of *EFR* tomato lines against global strains of *R. solanacearum* is necessary to evaluate their worldwide applicability for bacterial wilt management. The assessment of bacterial colonization following harvesting in bacterial wilt trials revealed that *EFR* and *Bs2/EFR* lines had a significantly reduced number of lower and middle stems with recoverable amounts of *R. solanacearum* compared with Fla. 8000 or the *Bs2* line (Table 5). Also, in the middle stem, no bacteria could be recovered from *EFR* lines or *Bs2/EFR* lines but viable bacterial populations were recovered from *Bs2* and nontransformed control

lines at 10^2 to 10^5 CFU/g of tissue (Fig. 1), thereby indicating *EFR*-based resistance is due to restricted bacterial colonization in upper stem tissues. In the bacterial spot trials, the reduction of bacterial spot severity achieved in *EFR* and *Bs2/EFR* transgenic lines over the nontransformed control did not seem to be affected by the zygosity of the *EFR* gene in the transgenic lines (Table 6). Boschi et al. (2017) also demonstrated similar observations in transgenic potato, where the copy number of *EFR* did not correlate with the response to bacterial wilt and genotypes with one, two, or three copies of *EFR* were equally responsive in disease reduction. Based on all of these results, it appears that *EFR* alone or in combination with *Bs2* could be used for integrated management of bacterial wilt and bacterial spot, two important diseases of tomato and other solanaceous crop.

Although transgenic expression of defense-related genes has been found to negatively impact yield in some crops (Chauhan et al. 2015; Risk et al. 2013), none of the studies relating to *EFR* transgenic crops, including tomato (Lacombe et al. 2010), wheat

TABLE 7. Comparison of tomato yield in nontransgenic tomato lines and transgenic tomato genotypes expressing *ELONGATION FACTOR TU RECEPTOR (EFR)* and *Bs2/EFR* in the field inoculated with *Xanthomonas perforans*^x

Entry ^z	Yield (kg/ha) ^y							
	Marketable				Total			
	Fall 2015	Spring 2016	Fall 2016	Spring 2017	Fall 2015	Spring 2016	Fall 2016	Spring 2017
FL47	24,025 d	65,657 bc	9,683 c	26,174 d	24,833 cd	70,043 abc	10,676 de	28,019 d
FL91	21,680 d	57,471 cd	9,285 c	49,481 c	21,925 d	58,832 cd	9,334 de	50,183 c
Sanibel	20,207 d	46,958 d	4,253 c	38,621 cd	20,787 d	49,304 d	4,428 e	38,970 cd
Fla. 8000	32,890 cd	64,259 bc	11,289 c	50,797 c	35,904 bc	68,777 bc	16,383 cd	54,185 bc
Fla. 8314	37,685 bc	67,004 abc	5,937 c	47,506 c	39,252 b	71,333 abc	6,511 de	49,343 c
Fla. 8000- <i>Bs2</i> **(<i>BC</i> ₄)	61,331 a	81,289 a	34,747 a	67,056 b	64,430 a	84,007 a	36,432 a	69,498 ab
Fla. 8314- <i>Bs2</i> *	62,605 a	75,857 ab	26,560 ab	72,062 ab	64,208 a	80,785 ab	28,481 ab	73,743 a
Fla. 8000- <i>EFR</i> **(<i>BC</i> ₆)	Nt	Nt	15,999 bc	45,583 c	Nt	Nt	16,993 bcd	47,122 c
Fla. 8000- <i>EFR</i> *(<i>BC</i> ₆)	Nt	Nt	24,743 ab	63,517 b	Nt	Nt	27,008 abc	66,971 ab
Fla. 8000- <i>Bs2</i> **/ <i>EFR</i> *(<i>BC</i> ₅)	46,982 b	68,788 abc	Nt	Nt	53,146 a	74,707 ab	Nt	Nt
Fla. 8000- <i>Bs2</i> **/ <i>EFR</i> *(<i>BC</i> ₆)	Nt	71,395 abc	30,434 a	71,479 ab	Nt	74,456 ab	34,105 a	73,381 a
Fla. 8000- <i>Bs2</i> **/ <i>EFR</i> **(<i>BC</i> ₆)	Nt	Nt	25,890 ab	72,126 ab	Nt	Nt	28,403 ab	74,718 a
Fla. 8314 <i>Bs2</i> */ <i>EFR</i> *(<i>BC</i> ₆)	Nt	Nt	Nt	81,212 a	Nt	Nt	Nt	82,132 a

^x Field trials were conducted fall 2015, spring 2016, fall 2016, and spring 2017 in Balm, FL. Column means followed by the same letter do not differ significantly at $P = 0.05$ based on least significant difference.

^y Marketable yield was recorded at harvest and calculated based on United States Department of Agriculture specifications by adding marketable (≥ 5.71 cm) fruit. Total yield was calculated by adding yield from marketable and small (< 5.71 cm) fruit. Marketable and total yield were analyzed separately for each year using one-way analysis of variance.

^z In the bacterial spot trials, each treatment in fall 2015 and fall 2016 consisted of three replications with 10 plants per replication and in spring 2016 and 2017 consisted of four replications with 8 and 10 plants per replication, respectively. Asterisks ** = homozygous and * = hemizygous, BC = backcross, and Nt = not tested. Fields were inoculated by spraying *Xanthomonas perforans* at 10^6 CFU/ml on the foliage of all plants with a backpack sprayer, applied along with polyoxyethylene sorbitan monolaurate (Tween 20; 0.05% vol/vol). Field trials were conducted using randomized complete block design.

TABLE 8. Summary of the bacterial spot field trial conditions and impact factor of *ELONGATION FACTOR TU RECEPTOR (EFR)* and *Bs2/EFR*^s

	Bacterial spot trial (Balm)			
	Fall 2015	Spring 2016	Fall 2016	Spring 2017
Temperature ^t	Normal, hot late	Normal	Normal, hot late	Normal
Rainfall ^u	Average, uneven	High-average, uneven	Low-average, uneven	Low-average, uneven
Soil temperature ^v	Normal, hot late	Normal	Normal	Normal
Relative humidity ^w	Normal, high late	Normal	Normal, low late	Normal
Disease pressure ^x	Medium	Low	High	High
Impact factor of <i>EFR</i> ^y	Nt	Nt	1.8	1.1
Impact factor of <i>Bs2/EFR</i> ^z	1.4	1.1	2.5	1.4

^s Bacterial spot trials were conducted during fall 2015, spring 2016, fall 2016, and spring 2017 in Balm, FL.

^t Normal: within 5% of typical averages; Nt = not tested.

^u Total rainfall relative to typical monthly totals.

^v Normal soil temperature is within 5% of typical averages.

^w Relative humidity is normal if the value is within 5% of typical averages.

^x Relative disease pressure based on bacterial spot severity rating. Low: $< 20\%$ disease severity in Fla. 8000, high: $> 60\%$ severity rating in Fla. 8000, and medium: 20 to 60% disease severity in Fla. 8000.

^y *EFR* impact factor was calculated by dividing the average marketable yield for Fla. 8000-*EFR* homo- and hemizygous plants with the marketable yield for Fla. 8000 plants.

^z *Bs2/EFR* impact factor was calculated by dividing the average marketable yield for Fla. 8000-*Bs2/EFR* (homozygous for *EFR*) and Fla. 8000-*Bs2/EFR* (hemizygous for *EFR*) with the marketable yield for Fla. 8000 plants.

(Schoonbeek et al. 2015), rice (Lu et al. 2015; Schwessinger et al. 2015), and potato (Boschi et al. 2017), has reported a detrimental effect of expressing *EFR*. In this study, despite the difference in weather and disease pressure across the two bacterial wilt trials, the *EFR* and *Bs2-EFR* gene increased marketable yield by 1.3- to 1.5-fold in 2015 and by 1.1- to 1.2-fold in 2016 relative to the Fla. 8000 control (Table 4). Also, in the bacterial spot trials, despite varying environmental conditions and disease pressure across trials, the *EFR* and *Bs2/EFR* lines displayed positive yield enhancement by 1.1- to 2.5-fold relative to the Fla. 8000 control (Table 8). Therefore, it is highly unlikely that *EFR* induces nonspecific constitutive defense or related trade-off on yield. However, it is important to consider that, in the bacterial wilt trials, the *EFR*- and *Bs2-EFR*-mediated increase in marketable yield was only marginal and statistically not significant, and only the yields for small fruit were significantly higher (data not shown). We speculate that the smaller fruit in the early (fourth) backcrosses of *EFR* and *Bs2/EFR* in the bacterial wilt trials are due to yield drag from Moneymaker, a small-fruited variety, which was used to introgress the *EFR* transgene into the *EFR* and *Bs2/EFR* transgenic lines. This was also evident in the bacterial spot trials where early backcrosses (BC₃ and BC₄) of *EFR* and *Bs2/EFR* were not found to improve marketable yield relative to the nontransformed Fla. 8000 control (data not shown). However, when late (sixth) backcrosses of *Bs2/EFR* and *EFR* (homozygous) were used, they significantly improved marketable yield relative to the nontransformed Fla. 8000 control in the majority (three of four) of trials (Table 7). Therefore, subsequent backcrosses of hemizygous and homozygous Fla. 8000-*EFR* and Fla. 8000-*Bs2/EFR* lines to the Fla. 8000 recurrent parent should improve fruit size while maintaining disease resistance. Further evaluation of *EFR* and non-*EFR* plants in noninoculated plots may have been helpful to confirm the negative trade-off, if any, of the *EFR* transgene on marketable yield of tomato. In this study, comparisons with noninoculated, disease-free plants were not possible because the field plots in the bacterial wilt trials had a previous history of *R. solanacearum*, and *X. perforans* is endemic under Florida field conditions. The overall yield of all of the treatments was relatively lower in 2016 than in 2015, despite lower disease pressure in 2016 than in 2015. This was because we had a major outbreak of *Tomato yellow leaf curl virus* (TYLCV) in 2016 at the bacterial wilt field trial site. Because both the Fla. 8000 lines, with or without *EFR*, are susceptible to TYLCV infections, a substantial amount of yield was lost from all plots in the 2016 trial, resulting in less yield in 2016 than in 2015. Although the reduction in bacterial spot severity was not affected by the zygosity of the *EFR* gene (Table 6) and, in most cases, the marketable yield was not affected by the zygosity of the transgene, the marketable yield in 2017 for the hemizygous *EFR* line (alone) compared with the homozygous *EFR* line (alone) was increased during bacterial spot trial (Table 7). In this case, *EFR* in the homozygous state appears to be detrimental to marketable yield compared with *EFR* in the hemizygous state. However, in this study, the homo- and hemizygous *EFR* lines were tested in only two of the bacterial spot trials and none of the bacterial wilt trials. Therefore, more field tests are necessary to elucidate the effect of zygosity of *EFR* genes on durability of resistance and on the marketable yield.

The results from this study further confirmed the ability of *Bs2* gene to improve marketable yield of tomato in fields infected with *X. perforans* (Horvath et al. 2012). However, unlike in bacterial spot trials, *Bs2* gene had no effect on yield in the field infected with *R. solanacearum*. This was expected because *R. solanacearum* lacks the *AvrBs2* effector, which is recognized by *Bs2* and triggers the defense response in the host (Horvath et al. 2012). *EFR* recognizes elongation factor Tu, a PAMP widely conserved across bacteria, and the *Bs2* protein recognizes the effector *AvrBs2*, which is highly conserved in *Xanthomonas* spp. The results of this study show that the stacking of *PRR* (such as *EFR*) and *R* genes (such as *Bs2*) might be a promising strategy to develop broad-spectrum

disease resistance in plants against many genera of plant-pathogenic bacteria, including bacterial wilt and bacterial spot of tomato—two of the most important bacterial diseases of tomato in Florida and worldwide.

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