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ORIGINAL ARTICLE

Linkage between the *I-3* gene for resistance to Fusarium wilt race 3 and increased sensitivity to bacterial spot in tomato

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Abstract

Key message **The negative association between the *I-3* gene and increased sensitivity to bacterial spot is due to linkage drag (not pleiotropy) and may be remedied by reducing the introgression size.**

Abstract Fusarium wilt is one of the most serious diseases of tomato (*Solanum lycopersicum* L.) throughout the world. There are three races of the pathogen (races 1, 2 and 3), and the deployment of three single, dominant resistance genes corresponding to each of these has been the primary means of controlling the disease. The *I-3* gene was introgressed from *S. pennellii* and confers resistance to race 3. Although *I-3* provides effective control, it is negatively associated with several horticultural traits, including increased sensitivity to bacterial spot disease (*Xanthomonas* spp.). To test the hypothesis that this association is due to linkage with unfavorable alleles rather than to pleiotropy, we used a map-based approach to develop a collection of recombinant inbred lines varying for portions of *I-3* introgression. Progeny of recombinants were evaluated for bacterial spot severity in the field for three seasons, and disease severities were compared between *I-3* introgression haplotypes for each recombinant. Results indicated that increased sensitivity to bacterial spot is not associated with the *I-3* gene, but rather with an upstream region of the introgression. A survey of public and private inbred lines and hybrids indicates that the majority of modern *I-3* germplasm contains a similarly sized introgression for which the negative association with bacterial spot likely persists. In light of this, it is expected that the development and utilization of a reduced *I-3* introgression will significantly improve breeding efforts for resistance to Fusarium wilt race 3.

Introduction

Fusarium wilt caused by the soil-borne fungus *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*), is a serious threat to field-grown tomatoes (*Solanum lycopersicum* L.) worldwide. The pathogen penetrates the root and infects the vascular system, causing yellowing, wilting, stunting, and death of the plant. Host resistance is the primary strategy for disease control, but in some areas, the adoption of the raised bed, plastic culture production system with soil fumigation has greatly improved management of *Fol*. Since the 1970s, much of the eastern US fresh-market tomato industry has heavily relied on the fumigant, methyl bromide, for management of nematodes, weeds, and soil-borne diseases—including Fusarium wilt. However, methyl bromide was found to be an ozone depleting substance by the Montreal Protocol in 1993, and for this reason, it is no longer produced or used as a soil fumigant in the US (Thomas 1996). Growers now rely on methyl bromide alternatives for fumigation, but under standard practices, these fumigants have been ineffective for managing Fusarium wilt (Vallad et al. 2014). In light of this,

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host resistance is becoming increasingly important for *Fol* disease management.

There are currently three races of *Fol*, and resistance to each of these is conferred by one of three single, dominant, resistance genes. The first of these genes to be discovered was termed *I* for "immunity," and was introgressed from *S. pimpinellifolium* for control of race 1 (Bohn and Tucker 1939). Resistance to *Fol* race 2 (*Fol2*) was also identified in *S. pimpinellifolium*, and this gene was subsequently characterized and named *I-2* (Alexander 1959; Stall and Walter 1965). Both *I* and *I-2* are located on chromosome 11, and both have been positionally cloned (Catanzariti et al. 2017; Simons et al. 1998). Resistance to race 3 (*Fol3*) was first reported in the *S. pennellii* accession PI414773, and the *I-3* gene from *S. pennellii* accession LA716 was subsequently characterized (McGrath et al. 1987; Scott and Jones 1989). Although *Fol3* resistance was originally thought to be conferred by *I-3* in both sources, it was later determined that a separate, *I-7* locus confers resistance in PI414773 (Lim et al. 2006). Gonzalez-Cendales et al. (2016) recently determined that *I-7* is located on chromosome 8, and they also cloned this gene.

Since its discovery, *I-3* has been the primary source of *Fol3* resistance for tomato breeding programs around the world. Although seedling disease screens are an effective strategy for identifying resistant plants, the use of linked co-dominant markers is preferred by breeders avoid the need to maintain pathogen cultures and conduct inoculations, as well as to improve selection efficiency by distinguishing between homozygous and heterozygous resistant plants. For much of the time since the discovery of *I-3*, resources for marker development were limited, but this is no longer the case. The first *I-3* marker to be used was identified by Bournival et al. (1989), who reported a 2.5 cM linkage distance between *I-3* gene and the *Got-2* isozyme marker on chromosome 7. Additional molecular markers more closely linked with *I-3* were developed from bacterial artificial chromosome (BAC) sequences by Barillas et al. (2008). Lim et al. (2006) mapped the *I-3* gene within a 0.57 cM region flanked by molecular markers CT226 and TG639, and *I-3* was later fine-mapped to a 0.38 cM (50–60 kb) interval flanked by the markers RGA332 and bP23/gPT (Lim et al. 2006, 2008). More recently, Catanzariti et al. (2015) cloned *I-3* and reported gene-specific markers. Yet throughout this period, there have been no reports of breeding programs utilizing a reduced *I-3* introgression relative to the size in donor materials originally available from the UF/IFAS tomato breeding program. Similarly, it has not been demonstrated that there is any practical benefit that may be realized by reducing the size of the *I-3* introgression.

Tomato cultivars containing *I-3* have been commercially available since the early 1990s. However, development of such hybrids has been, and continues to be, challenging due to the negative association of *I-3* with several horticultural traits. Scott (2004) reported that some of the earliest *Fol3* resistant parent lines had a higher incidence of blossom end rot than lines that did not contain *I-3*, and this trend is occasionally observed in modern *I-3* materials (R. A. Gardner, personal communication). It was also reported that tomato breeding lines homozygous for *I-3* had smaller fruit size compared to plants heterozygous for *I-3* (Scott 1999). This effect was recently confirmed in each of two University of Florida breeding line backgrounds, Fla. 7907B and Fla. 8814. In that study, *I-3* had a dosage effect on fruit size, where heterozygosity for *I-3* resulted in intermediate fruit size relative to homozygous *I-3/I-3* and homozygous *i-3/i-3* plants, with homozygous resistant plants having the smallest fruit size (Hutton, unpublished data). Besides this, Hutton et al. (2014) showed that the *I-3* locus is also associated with a greater sensitivity to bacterial spot disease. They found that homozygous *I-3* plants were more severely infected with bacterial spot (up to 20% more disease compared with plants homozygous for the susceptible allele), and plants heterozygous for *I-3* again had an intermediate response relative to homozygous genotypes.

Bacterial spot is a serious disease in many tomato production regions with warm and humid climates. Four *Xanthomonas* species cause bacterial spot in tomato, i.e., *X. euvesicatoria* (race T1), *X. vesicatoria* (race T2), *X. perforans* (races T3 and T4), and *X. gardneri* (Jones et al. 2000, 2005). The pathogen infects most above-ground parts of tomato plants, including leaves, stems, and fruits. It causes necrotic lesions and defoliation and results in defoliation that can severely impact total yield and fruit quality (Scott and Jones 1986). Pohronezny and Volin (1983) reported that total yield reduction caused by bacterial spot could range from 22 to 52% compared with non-inoculated control plants. Because both bacterial spot and Fusarium wilt race 3 are significant disease problems in Florida and in many other tomato production regions of the world, it would be useful to determine whether the cause of this association is due to linkage with undesirable genes, or to a pleiotropic effect of the *I-3* gene itself.

In this study, we use a map-based approach to test the hypothesis that the association between increased bacterial spot sensitivity and *Fol3* resistance is due to linkage drag and that this linkage might be broken. Our objectives are: (1) to ascertain *I*-3 introgression sizes in a collection of modern germplasm; and (2) to develop and test a series of recombined *I-3* introgressions for each's effect towards bacterial spot sensitivity.

Materials and methods

I-3 introgression sizes within modern germplasm

To initially ascertain the size of the *I*-3 introgression in modern germplasm, whole genome re-sequencing was conducted for the *Fol3*-resistant inbred line, Fla. 630 (described below). Parents of Fla. 630 include Fla. 8517 (Hutton et al. 2010) and the *I-3* inbred, Fla. 7946 (Scott 2004). Subsequently, to compare introgression sizes among modern germplasm and to validate newly developed molecular markers, a collection of inbred lines and hybrids with known resistance or susceptibility to *Fol3* were obtained from multiple public and private breeding programs (Table S1). *S. pennellii* accession LA716 was obtained from the Tomato Genetics Resource Center in Davis, CA and used for comparison. The majority of the inbred lines were from the University of Florida, Institute of Food and Agricultural Sciences (UF/IFAS) tomato breeding program. In addition to these, five inbred lines were provided by D.M. Francis at the Ohio State University, two inbred lines were provided by R. A. Gardner and D. Panthee at North Carolina State University, and the remaining inbred lines were openly available. Of the 26 hybrids surveyed, one was provide by R. A. Gardner and D. Panthee at North Carolina State University, one was provided by Bejo Seeds, Inc., three by Enza Zaden, two by Hazera, two by HM Clause, six by Sakata, six by Seminis, and five by Syngenta. Each of these lines was genotyped with 13 of the *I-3* introgression markers described below.

DNA extraction, whole genome re-sequencing, and molecular marker analyses

Fla. 630 seedlings were grown in a greenhouse, and DNA was extracted using the CTAB method (Doyle 1987), yielding about 500 ng/µl of genomic DNA for whole genome re-sequencing. Paired-end libraries of Fla. 630 were generated and sequenced on an Illumina HiSeq 2000 machine at the Weil-Cornell Genomics Core Facility, New York, NT. Each PE library had an insert size of 300 bp. The reference genome for *S. lycopersicum* H1706 used for alignment is from the international tomato genome project, version SL2.50 (http://solgenomics.net/organism/Solanum_lycopersicum/genome). SNPs and indels of Fla. 630 were called in reference to the H1706 tomato genome. SNPs and Indels that were not unique to *S. pennellii* were filtered out by removing ones shared with the *Fol3*-susceptible heirloom line Yellow Pear (Strickler et al. 2015). SNPs and Indels in each chromosome were aggregated into bins of 10 Kb (https://github.com/nmenda/GenomeTools; Menda et al. 2014). The unique SNPs and indels were plotted using R statistics (http://www.R-project.org) to visualize the locations where putative genome introgressions can be detected. The genome of Fla. 630 is available to browse, BLAST and download at the Sol Genomics Network website (http://solgenomics.net) and through Genbank (accession PRJNA413174).

For marker screens, DNA samples were extracted from individual seedlings using a microprep protocol described by Fulton et al. (1995). For all marker assays, the polymerase chain reaction (PCR) protocol for Phire® Hot Start II DNA polymerase (Thermo Scientific) was used. Reactions were performed in a 10 µl volume for each sample, which included 2 µl of DNA (approximately 20 ng/µl concentration), 4.89 µl of H_2O, 2 µl of 5× Phire Reaction Buffer, 0.8 µM dNTPs, 0.4 µM each of forward and reverse primers, and 0.25 units of polymerase. For CAPS markers, PCR products were digested with the appropriate restriction enzyme according to the manufacturer's instructions. Genotypic results were detected using electrophoresis on 3% agarose gels stained with ethidium bromide. CAPS markers were obtained from Lim et al. (2008). Sequence characterized amplified region (SCAR) markers were designed by comparing the *S. lycopersicum* genome assembly SL2.50 (Tomato Genome Consortium 2012) and *S. pennellii* genome assembly (Bolger et al. 2014) to identify genomic insertion/deletion (Indel) mutations. Primer 3 software (Koressaar and Remm 2007) was then used to design PCR-based markers from flanking sequences of target indels, resulting in the development of 13 additional markers which were validated on the germplasm described above (Table S2).

Bacterial spot trials of *I-3* introgression recombinants

Two *Fol3* resistant recombinant inbred lines (RILs) in the *S. lycopersicum* cv. M82 background and containing overlapping *I-3* introgressions derived from *S. pennellii* accession LA716 were developed by Lim et al. (2008) and obtained from D. A. Jones at the Australian National University. One RIL (hereafter termed as *I-3*-1) contains the *S. pennellii* introgression spanning from the top of the chromosome down to just below the *I-3* gene, while the second (hereafter termed as *I-3*-2) contains the *S. pennellii* introgression spanning from the bottom of the chromosome up to just above the *I-3* gene. Both RILs were backcrossed four times to the *Fol3* susceptible breeding lines Fla. 7781 and Fla. 8059 to develop near isogenic lines (NILs) for testing the effect of these introgressions within Florida-adapted backgrounds (Scott and Jones 2000; Scott et al. 2008). These NILs were confirmed by *Fol3* seedling disease screens to have *Fol3* resistance, and they were also genotyped with several *I-3* molecular markers to more precisely determine the boundaries of the *I-3* introgression in each. NILs containing the *I-3* introgressions derived from *I-3*-1 and *I-3*-2 were evaluated for bacterial spot disease severity in spring 2014 and fall 2014 field trials. Spring trials were planted on April 9th 2014, and disease severity was evaluated on June 3rd. For

fall trials, planting was on August 28th 2014, and disease severity was evaluated on November 18th. Controls for these trials included: M82 in the spring; and M82, *I-3*-1 and *I-3*-2, Fla. 8059 and Fla. 7781 in the fall. For each trial, a randomized complete block design (RCBD) was used with three blocks and six plants per plot.

Florida 7228 is a large-fruited, fresh-market tomato breeding line with *Fol3* resistance derived from *S. pennellii* accession LA716. This breeding line resulted from three crosses into cultivated tomato, and it served as the *I-3* donor in the development of the released *Fol3* resistant breeding lines, Fla. 7547 and Fla. 7481 (Scott and Jones 1995). Florida 7228 was crossed to each of the *Fol3* susceptible breeding lines, Fla. 7169, Fla. 7171 and Fla. 7217D. For each cross, F_1s were allowed to self-pollinate to produce F_2 seed, and F_2 plants heterozygous for the *I-3* introgression (*I-3*/*i-3*) were then self-pollinated to produce seed for an F_3 population. F_3 seedlings were screened for recombination within the *I-3* introgression interval spanned by the markers *I-3CAPS*, *SLG-1* and *bB6*. Recombinants were selected, and progeny of each were evaluated for bacterial spot disease severity over three production seasons. For spring 2014 and spring 2015 trials, seedlings were genotyped with markers corresponding to the segregating portion of their *I-3* introgression, transplants were grouped according to genotype at these markers (homozygous *S. pennellii*, heterozygous, or homozygous *S. lycopersicum*), and the homozygous haplotypes of each recombinant were used for field trials. In spring 2014, $F_{3:4}$ seed were sown on February 6th. During that season, $F_{3:5}$ seed was bulk-harvested from each haplotype of each recombinant, and $F_{4:5}$ seed was also harvested from a single plant of each recombinant's heterozygous group. The bulk-harvested $F_{3:5}$ seed was sown on July 10th, 2014 for the fall 2014 trial, and the $F_{4:5}$ seed was sown on March 26th, 2015 for the spring 2015 trial. Controls for these trials included the parental lines Fla. 7228, Fla. 7169and 7217D. A RCBD with three blocks and six plant plots was used for each trial except spring 2015, which had four plants per plot.

All field trials were conducted at the Gulf Coast Research and Education Center in Balm, FL. For each of these trials, seed was sown directly into peat-lite soilless media (Speedling, Sun City, FL, USA) in 128-cell Speedling® trays (38 cm^3 cell size; Speedling). Transplants were grown in a greenhouse and planted to field beds approximately 5–6 weeks after sowing. Field beds were 20 cm high and 81 cm wide and had been fumigated with a combination of chloropicrin and 1, 3-dichloropropene (Pic-Clor 60 EC, Soil chemical corporation, Hollister, CA, at 300 lbs per treated acre) and covered with black or white plastic mulch (for spring or fall trials, respectively) before transplanting. Beds were spaced 152 cm apart, and transplants were planted in a single row within each bed. In-row plant spacing was 46 cm. The tomato plants were staked and tied, and irrigation was applied through drip tape beneath the plastic mulch of each bed. A recommended fertilizer and pesticide program was followed throughout the growing season, excluding the use of SAR inducers, copper, and other bactericides (Freeman et al. 2015).

Field trials were inoculated approximately 3 weeks after transplanting. Inoculum was prepared by growing *X. perforans* race T4 on Difco nutrient agar (Becton–Dickinson and Company, Sparks, MD) for 24–36 h at 28 °C. Bacterial colonies were removed from the agar plates and suspended in 10 mM $MgSO_4 \cdot 7\ H_2O$. The inoculum was standardized to $A_{600} = 0.30$ (a concentration of approximately $2–5 \times 10^8$ colony forming units per ml). Inoculum was applied to plants by misting the foliage with a backpack sprayer. Foliar disease severity was rated using the Horsfall and Barratt scale (1945), where 1 = 0%, 2 = 0–3%, 3 = 3–6%, 4 = 6–12%, 5 = 12–25%, 6 = 25–50%, 7 = 50–75%, 8 = 75–87%, 9 = 87–94%, 10 = 94–97%, 11 = 97–100%, and 12 = 100% diseased tissue (Fig. S1). Ratings were scored on individual plants and averaged across plants within each experimental unit. For presentation purposes, disease severity ratings were converted to percent disease using midpoints of the ranges described above. Disease severity data were analyzed using a nonparametric procedure for the analysis of ordinal data (Brunner and Puri 2001; Shah and Madden 2004). The overall effect of the different *I-3* introgression sizes on bacterial spot sensitivity within each NIL or RIL was analyzed by analysis of variance type statistic of ranked data using the PROC MIXED procedure in SAS (version 9.4; SAS Institute, Cary, NC, USA). Relative marginal effects (RME) were generated with the equation: $RME = (R - 0.5)/N$; where R is the mean treatment ranking and N is the total number of experimental units in the analysis, and the LD_CI macro was used to generate 95% confidence intervals (Brunner and Puri 2001; Shah and Madden 2004). Blocks were nested within seasons, and both block and season were considered random effects.

Fol3 disease screens

To confirm presence of *I-3* in the *I-3*-1 and *I-3*-2 NILs, and to determine the presence/absence of *I-3* in each of the Fla. 7228-derived recombinants, seedling disease screens were conducted. Screens used the *Fol3* strain, GEV 1400 Pf120210F (provided by the University of Florida Plant Pathology lab at the GCREC). The fungus was grown on potato dextrose agar (PDA) media at 28 °C for approximately 1 week. Inoculum was prepared by removing the fungus from the agar into 100 ml DI water, and adjusting the suspension of fungal conidia to approximately 10^7 spores per ml. For the Fla. 7228-derived recombinants, recombinant inbred lines (RILs) were developed using seed that was bulk harvested in spring 2014 from one of the homozygous

genotypic groups. Seed was sown in Black Beauty spent coal (Harsco Minerals International, Mechanicsburg, PA, USA) in a growth room, where seedlings were maintained until inoculation. Sixteen seedlings per RIL/NIL were inoculated at approximately 2 weeks after sowing, transplanted into peat-lite soilless media in 128-well Speedling® trays, and then maintained in a greenhouse until disease development. Each tray included a minimum of four plants of each of the control lines, Fla. 7547 and 'Horizon' (Scott et al. 1985). Seedlings were inoculated by gently removing them from the coal, then dipping the roots into the conidia suspension for 15 s. Plants were evaluated approximately 3 weeks after inoculation as described previously (Scott et al. 2004). Briefly, plants that were dead or showed signs of stunting, yellowing, wilting, enlarged stem, and stem collapse were considered susceptible. For questionable plants, the lower part of the stem was dissected to determine the presence of vascular browning, which is indicative of *Fol3* infection. Symptomless, healthy plants were considered resistant.

Results

Whole genome re-sequencing of Fla. 630 and plotting of SNP and indel frequency along chromosome 7 clearly indicated the presence of a wild species introgression at the lower end of the chromosome (Fig. S2). The introgression is marked by an increase in polymorphism frequency from 59.717 to 63.774 Mbp on the SL2.50 tomato genome assembly. A separate peak between 67.11 and 67.16 Mbp was also observed in Fla. 630. However, this potential introgression is not directly related to *I-3*, as other re-sequencing efforts have demonstrated its presence in a number of *Fol3* susceptible UF/IFAS breeding lines (Hutton, unpublished data). The identification of the ≈ 4 Mb *S. pennellii* introgression, together with *I-3* fine-mapping results of Lim et al. (2008), was used to guide subsequent marker development efforts.

To assess *I-3* introgression sizes within commercial germplasm and to validate the utility of newly developed molecular markers for distinguishing resistant and susceptible alleles across various backgrounds, we used 13 *I-3* markers to survey a collection of 71 hybrids and inbred lines obtained from public and private breeding programs. Results are presented in Tables 1 and S1. Each of the *I-3* markers is co-dominant and consistently distinguished the *S. pennellii* allele from the *S. lycopersicum* allele across the range of market types and germplasm sources tested. One marker, *7g665* distinguished two different *S. lycopersicum* alleles, but each of these was distinguishable from the resistant allele. Many of the *Fol3* resistant breeding lines and hybrids contained an *I-3* introgression that was identical in size to that of Fla. 630, spanning markers *7g6096* to *bB6* (Groups 2 and 4). Germplasm containing this ≈ 4 Mb introgression included inbred lines from UF/IFAS and hybrids from Enza Zaden, Hazera, HM Clause, Sakata, Seminis and Syngenta. Some of the *Fol3* resistant lines, however, had one of three differently sized introgressions. Florida 7228 was among these, and it had the largest introgression which spanned more than 5 Mb from *bB6* on the lower end to beyond *7g5862* on the upper end. Inbred lines in Group 1 (Fla. 7481, Fla. 7907B and NC123S) and hybrids in Group 3 ('NC 0256' and Tasti-Lee®) all contained a second introgression size that is between 4.2 and 5.1 Mb and ends between markers *7g5951* and *7g5862*. Lastly, a smaller introgression was found in Group 5 hybrids ('Skyway 687' and 'Pawnee'). In both of these, the upper boundary of the introgression was between *7g6102* and *I-3CAPS*, resulting in an introgression that is between 1.0 and 2.7 Mb in size.

Table 1 Genotypic results for *I-3*-linked markers among 71 public and private tomato inbred lines and hybrids

Lines	Markers			
	7g5862	*7g5951*	*7g6096/7g6102*	*I-3CAPS*[a]
Fla. 7228	PP[b]	PP	PP	PP
Group 1[c]	LL	PP	PP	PP
Group 2	LL	LL	PP	PP
Group 3	LL	LP	LP	LP
Group 4	LL	LL	LP	LP
Group 5	LL	LL	LL	LP
Group 6	LL	LL	LL	LL

[a]Plus downstream introgression markers (see Table S1)

[b]Marker genotypes: PP indicates homozygosity for the *S. pennellii* allele; LL indicates homozygosity for the *S. lycopersicum* allele

[c]See Table S1 for details of lines tested within each group

To initially test whether the association between the *S. pennellii I-3* introgression and increased bacterial spot sensitivity may be the result of linkage drag, we evaluated near isogenic lines (NILs) containing either of two different *I-3* introgressions in each of three recurrent parent (RP) backgrounds, M82, Fla. 8059, and Fla. 7781. Both introgressions overlap at the *I-3* locus, at marker *SLG-1* (Lim et al. 2008). The *I-3*-1 introgression is very large and extends from the beginning of chromosome 7 to just beyond the *I-3* locus. Contrastingly, the *I-3*-2 introgression extends from just above the *I-3* locus down to the end of the chromosome (Table 2). NILs were evaluated in spring and fall 2014, and results are presented in Table 2. In the M-82 background, disease severity relative to the RP (RME = 0.50) was significantly increased by presence of the *I-3*-1 introgression (RME = 0.90). In the Fla. 8059 and Fla. 7781 backgrounds, the *I-3*-1 NILs likewise had higher RMEs than their respective RPs, but these differences were not significant. For all three backgrounds, the *I-3*-1 NILs had significantly higher RMEs than the *I-3*-2 NILs. Presence of the *I-3*-2

Table 2 Bacterial spot race T4 disease severity comparisons among near isogenic lines (NILs) containing either of two different *Solanum pennellii* chromosome 7 introgressions relative to the recurrent parent, in each of three inbred backgrounds of tomato

Lines	Genotypes at markers defining the *I-3* introgression[a]						Disease severity (%)[b]	Rank		Effect	
	I-3CAPS	*7g916*	*7g923*	*SLG-1*	*7g957*	*bB6*		Median	Mean	RME[c]	95% CI
			I-3								
I-3-1	*PP*	*PP*	*PP*	*PP*	*LL*	*LL*	63	15.0	15.1	0.90	0.80–0.92
I-3-2	*LL*	*LL*	*PP*	*PP*	*PP*	*PP*	43	8.5	7.3	0.58	0.41–0.72
M82	*LL*	*LL*	*LL*	*LL*	*LL*	*LL*	40	5.5	6.1	0.50	0.34–0.67
I-3-1-8059	*PP*	*PP*	*PP*	*PP*	*LL*	*LL*	43	11.0	10.3	0.59	0.41–0.73
I-3-2-8059	*LL*	*LL*	*PP*	*PP*	*PP*	*PP*	31	4.8	4.5	0.25	0.15–0.41
Fla. 8059	*LL*	*LL*	*LL*	*LL*	*LL*	*LL*	34	11.0	10.0	0.45	0.29–0.62
I-3-1-7781	*PP*	*PP*	*PP*	*PP*	*LL*	*LL*	39	9.5	9.8	0.45	0.30–0.61
I-3-2-7781	*LL*	*LL*	*PP*	*PP*	*PP*	*PP*	28	4.8	5.8	0.23	0.13–0.43
Fla. 7781	*LL*	*LL*	*LL*	*LL*	*LL*	*LL*	35	9.5	7.4	0.31	0.13–0.63

[a]Introgression size in each NIL, where "*PP*" indicates homozygosity for *S. pennellii I-3/I-3* allele; "*LL*" indicates homozygosity for *i-3/i-3* susceptible allele

[b]Mean disease severity averaged across two field seasons

[c]Estimated relative marginal effect (RME) and 95% confidence intervals (CI) calculated from analysis of the rank values of the disease severity data. RMEs are compared within each background

introgression did not increase bacterial spot disease severity for any of the backgrounds.

Toward determining what effect reducing the size of the *I-3* introgression may have on bacterial spot sensitivity, we developed a series of *I-3* recombinants using germplasm adapted to Florida's environmental conditions. Fla. 7228 was used as the *I-3* donor in crosses with three *Fol3* susceptible breeding lines, and F_3 populations were produced from these crosses and screened for recombination within the one Mb interval between 62.7 and 63.7 Mb. A total of 2820 F_3 plants were genotyped, and 15 plants resulting from cross-over events were identified and selected, hereafter referred to as "R##" (e.g., R19, R23, etc.). Each recombinant was self-pollinated, and $F_{3:4}$ plants homozygous for the recombined introgression were selected and genotyped with all *I-3* markers. Crossing-over was not evenly distributed throughout the interval, but occurred more frequently in the ≈ 290 Kb interval below 63.41 Mb (13 recombination events between markers *7g728* and *bB6*) than in the ≈ 640 Kb interval above 63.41 Mb (two recombination events between markers *I-3CAPS* and *7g728*; Fig. 1). To confirm the presence or absence of *I-3*, $F_{3:5}$ recombinant inbred lines (RILs) for each *I-3* recombinant were subjected to seedling *Fol3* disease screens. Ten of the RILs were resistant, and five were susceptible, and *Fol3* response corresponded to an approximately 30 kb interval between markers *7g916* and *7g923* (data not shown). This position is in complete accord with the known location of the *I-3* gene (Catanzariti et al. 2015).

We evaluated progeny of each Fla. 7228-derived recombinant for bacterial spot sensitivity in the field for three consecutive production seasons. For each recombinant, disease severities were compared between the *I-3* haplotypes to determine the effect of different introgression sizes toward bacterial spot sensitivity. Results are presented in Table 3 and summarized in Fig. 1. The *I-3* control line, Fla. 7228, was significantly more sensitive to bacterial spot than either of the *Fol3* susceptible controls tested. Among the recombinant lines, no significant differences in mean disease severity rankings were found between haplotypes of RILs containing portions of the distil part of the *S. pennellii* introgression at and below the *I-3* gene. This held true for those recombinant lines that did not segregate for *I-3* (R11, R17, R12, R19 and R23), as well as for those that did segregate for *I-3* (R10, R18 and R26). In contrast, significant differences in the mean disease severity rankings were observed between haplotypes of all but one (R024) of the recombinant lines containing portions of the proximal part of the *S. pennellii* introgression. Among these recombinants, the introgressions extended as far down as marker *7g957* (in the case of R14), or ended as high up as marker 7g728 (in the case of R09). Again, significant differences were observed for recombinant lines that did segregate for *I-3* (R14 and R15), as well as for lines that did not segregate for *I-3* (R08, R09, R07, and R21). In each case, the *S. pennellii* haplotype demonstrated a greater disease severity ranking relative to the *S. lycopersicum* haplotype. This trend was also similar, although not significant, for R24, which had a slightly higher mean disease severity and mean ranking for its *S. pennellii* haplotype than its *S. lycopersicum* haplotype. These results provide compelling evidence that increased sensitivity to bacterial spot is not

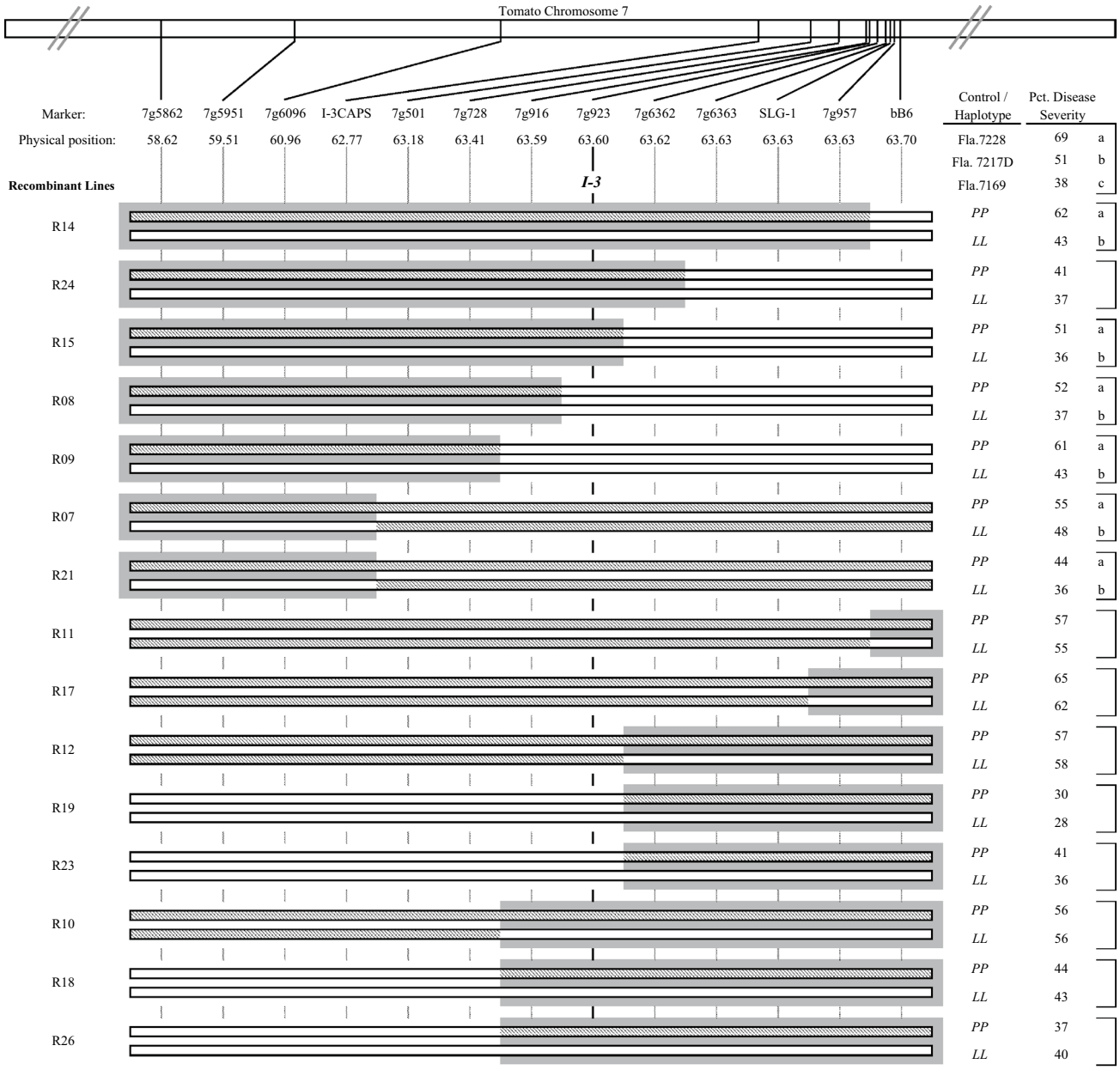

Fig. 1 Bacterial spot race T4 disease severity comparisons between recombinant and non-recombinant haplotypes of fifteen *I-3* recombinant lines differing for portions of the *Solanum pennellii* introgression on chromosome 7. Physical positions (Mb) are presented under each marker name and correspond to the SL2.50 tomato genome assembly (Tomato Genome Consortium 2012). Diagrams of the *I-3* introgression region for each recombinant depict each chromosome, and introgressed segments of the *S. pennellii* genome are represented by diagonal stripes. The gray-shaded regions indicate the portion of the introgression that segregated for either haplotype of each recombinant. The location of the *I-3* gene is indicated by the thick, vertical line. Disease severity is presented as percent infected tissue and corresponds to the average severity across three field seasons. Comparisons were made among controls and between haplotypes of each recombinant (not among recombinants). Fla.7228 was included as an *I-3* control, and Fla.7217D and Fla.7169 were *Fol3*-susceptible controls. Haplotype designations correspond to their genotype in the gray-shaded regions (*PP* for homozygosity of the *S. pennellii* alleles, and *LL* for homozygosity of the *S. lycopersicum* alleles). Means followed by different letters are significantly different ($P < 0.05$) based on ranked mean analyses

directly associated with *I-3*, but rather with a region of the *S. pennellii* introgression above *I-3*, and most likely above marker *7g501*.

Discussion

In this study, we screened nearly 3000 plants segregating for a > 5 Mb *S. pennellii I-3* introgression to identify

Table 3 Median and mean rankings and relative marginal effect (RME) calculated for bacterial spot disease severity on tomato controls and on recombinant and non-recombinant haplotypes for each of fifteen recombinants differing for portions of the *Solanum pennellii* introgression on chromosome 7

Line	Haplotype[a]	Rank		Effect	
		Median	Mean	RME	95% CI
Fla.7228	Control	15.5	15.3	0.82	0.75–0.83
Fla.7217D	Control	7.5	7.5	0.39	0.29–0.52
Fla.7169	Control	6.0	5.6	0.28	0.21–0.43
R14	*PP*	13.0	12.3	0.66	0.51–0.72
	LL	4.5	6.7	0.34	0.28–0.49
R24	*PP*	8.0	10.2	0.54	0.40–0.66
	LL	8.0	8.8	0.46	0.34–0.60
R15	*PP*	14.5	13.0	0.69	0.57–0.74
	LL	5.5	6.0	0.31	0.26–0.43
R08	*PP*	11.5	11.9	0.64	0.49–0.71
	LL	7.0	7.1	0.36	0.29–0.51
R09	*PP*	14.5	13.1	0.70	0.51–0.74
	LL	4.0	5.9	0.30	0.26–0.49
R07	*PP*	8.3	7.7	0.60	0.42–0.71
	LL	4.5	5.3	0.40	0.29–0.58
R21	*PP*	14.0	11.7	0.62	0.47–0.70
	LL	9.0	7.3	0.38	0.30–0.53
R11	*PP*	11.0	9.7	0.51	0.38–0.64
	LL	8.0	9.3	0.49	0.36–0.62
R17	*PP*	10.0	10.2	0.54	0.40–0.65
	LL	10.0	8.8	0.46	0.35–0.60
R12	*PP*	11.0	9.5	0.50	0.37–0.63
	LL	11.0	9.5	0.50	0.37–0.63
R19	*PP*	8.3	8.8	0.58	0.40–0.69
	LL	8.0	7.5	0.42	0.31–0.60
R23	*PP*	9.5	11.1	0.59	0.45–0.68
	LL	9.5	7.9	0.41	0.32–0.68
R10	*PP*	12.5	9.6	0.51	0.38–0.63
	LL	9.0	9.4	0.49	0.37–0.62
R18	*PP*	9.5	9.6	0.51	0.38–0.63
	LL	9.5	9.4	0.49	0.37–0.62
R26	*PP*	8.0	8.8	0.45	0.35–0.59
	LL	8.0	10.2	0.54	0.41–0.65

[a]Haplotype designations for each recombinant correspond to their genotype in the segregating portion of the introgression: *PP* for homozygosity of the *S. pennellii* alleles, and *LL* for homozygosity of the *S. lycopersicum* alleles

recombinants useful for reducing the introgression size. To focus our efforts on recombination events occurring close to the *I-3* gene, we used the markers *I-3CAPS* and *bB6*, which flank the fine-mapped *I-3* interval (Lim et al. 2008). Our screening recovered 15 recombinants for this ≈ 1 Mb interval, which corresponds to 0.53 cM. According to the high-density linkage map for the EXPEN 2012 interspecific F_2 population (*S. lycopersicum* Moneymaker × *S. pennellii* LA0716), the *I-3CAPS* and the *bB6* markers correspond to 43.4 and 45.0 cM, respectively, or 1.6 cM apart (Sim et al. 2012). Similarly *I-3CAPS* and *bB6* are located 2.8 cM apart at 50.0 and 52.8 cM, respectively, on the EXPEN 2000 linkage map generated by Fulton et al. (2002) using a different interspecific F_2 population (*S. lycopersicum* LA925 x *S. pennellii* LA0716). Thus, the realized rate of recombination from our screening is between one-third and one-fifth of the rate that would have been expected based on these linkage maps. Such suppression of recombination is typical within introgressions from wild species, and suppression within *I-3* introgressions has been reported previously (Hemming et al. 2004; Houterman et al. 2008). Lim et al. (2008) compared rates of recombination for three different populations, each segregating for a differently sized *I-3* introgression. They observed that the intensity of recombination suppression increases as the introgression size decreases. The interval of comparison in their study corresponds to the region between markers *7g501* and *bB6* in our population. For this interval, we observed a recombination frequency of 13/2820 (or 1/217), which is similar to the 1/172 frequency Lim et al. (2008) described for the (M82 × IL7-3) population.

Phenotypic analysis of our recombinants for resistance to *Fol3* delimited *I-3* to a < 30 kb region. This region contains four annotated genes, one of which is the S-receptor-like kinase identified by Catanzariti et al. (2015) as the *I-3* gene. For practical breeding purposes, markers located within or extremely tightly linked to a gene are preferred. Catanzariti et al. (2015) described two CAPS markers that are each located within the *I-3* gene and should be useful for selection purposes. Of the SCAR markers developed in our study, *7g923* is likewise located within the *I-3* gene and may be useful to breeding programs interested in utilizing a SCAR marker to select for the *I-3* gene.

Both bacterial spot and *Fol3* are major diseases in many warm, humid production regions of the world, including Florida. There are currently no commercially available bacterial spot resistant tomato cultivars, and neither are there effective control strategies for this disease. Hutton et al. (2014) previously reported that increased sensitivity to bacterial spot is associated with resistance to *Fol3* conferred by *I-3*. This association is particularly challenging for breeders in areas such as Florida, since cultivars are needed which provide tolerance to bacterial spot as well as resistance to *Fol3*. It was not known, however, whether this association resulted from a pleiotropic effect of the *I-3* gene, or from tightly linked negative alleles within the *S. pennellii* introgression. To address this question, we compared bacterial spot sensitivity among haplotypes of multiple *I-3* introgression recombinants over three seasons. Our results provide compelling evidence that the association between *Fol3* resistance and increased bacterial spot sensitivity is not due

to pleiotropy, but rather results from negative loci located upstream of *I-3*.

To assess the relevance of this finding for active tomato breeding programs, we surveyed a collection of inbreds and hybrids from public and private breeding programs for *I-3* introgression sizes. Our survey included *Fol3* resistant germplasm from the public tomato breeding programs at University of Florida (UF/IFAS) and North Carolina State University, and from private breeding programs, including Bejo, Enza Zaden, Hazera, HM Clause, Sakata, Seminis and Syngenta. A ≈ 4 Mb introgression size was observed in the majority of commercial hybrids and in most UF/IFAS inbred lines, including *I-3*R1 and Fla. 7547. This is not surprising, considering that germplasm developed in the UF/IFAS tomato breeding program was the original source of *I-3*, and that *I-3*R1 and Fla. 7547 were widely distributed and utilized by many tomato breeding programs. The largest *I-3* introgression, observed in Fla. 7228, was > 5 Mb. Several lines contained an introgression smaller than that in Fla. 7228, but larger than the ≈ 4 Mb introgression. These include UF/IFAS inbred lines Fla. 7481 and Fla. 7907B; NCSU lines NC 123S and NC 0256; and the Bejo hybrid, Tasti-Lee® (a.k.a., Fla. 8153). Florida 7481 and Fla. 7547 are nearly isogenic lines with *Fol3* resistance derived from Fla. 7228, so the introgression was obviously reduced during the development and selection of these inbreds (Scott and Jones 1995). But although these would be hypothesized to have similar introgression sizes, Fla. 7547 evidently resulted from an additional recombination event shortening its introgression. This is confirmed by the findings of Lim et al. (2008), who likewise reported a larger introgression in Fla. 7481 than in Fla. 7547. The presence of this larger introgression in NCSU germplasm is explained by the fact that much of the *Fol3* resistant material in the NCSU breeding program traces to Fla. 7481, which is the case for NC 123S and NC 0256 (R. A. Gardner, personal communication). Likewise, Fla. 7907B is the *Fol3* parent in Fla. 8153, and the *I-3* introgression in Fla. 7907B traces directly to a sister line to Fla. 7481 (Scott et al. 2008).

The negative association between the *I-3* locus and increased bacterial spot sensitivity was originally observed in populations developed using Fla. 7946 and segregating for the ≈ 4 Mb introgression (Hutton et al. 2014). Because most of the modern *Fol3* resistant germplasm screened in this study contains a similar or larger introgression, it is apparent that the introgression size in at least some modern commercial germplasm has not been reduced, and that both public and private breeding programs may benefit from the utilization of a smaller *I-3* introgression. Notably, two Enza Zaden hybrids, 'Skyway 687' and 'Pawnee,' each contain a smaller introgression that is between 1.0 and 2.7 Mb in size. The results of our study demonstrate that the bacterial spot sensitivity locus is above marker *7g501*, but its precise position is not known. Whereas it is possible that the introgression in the Enza Zaden hybrids is not associated with this locus, it is also possible that the negative allele(s) is located nearer to the *I-3CAPS* marker and is still present in the Enza Zaden germplasm. Further testing of populations developed from these hybrids would be needed to determine which is the case.

Our findings prompt the question as to why, in light of the associated linkage drag and after more than 20 years of continuous breeding efforts, has the introgression not been further reduced. The suppression of recombination within the introgression provides one explanation, since useful recombinants would be quite rare. Together with this, three things should also be considered. First, that the effect of the introgression on bacterial spot sensitivity is subtle and was only recently documented. Hutton et al. (2014) reported that differences in average bacterial spot disease severity ranged from 2 to 20% between *Fol3* homozygous resistant and homozygous susceptible plants, and differences between these and heterozygous classes was even less. Second, such rare recombinants would have to be evaluated in an environment with sufficient bacterial spot pressure to allow the recognition of these subtle differences in disease sensitivity. And importantly, the recombination event must have also occurred in a plant that otherwise had sufficient horticultural performance for it to be selected and advanced. It is thus understandable how useful recombinants might go unnoticed by breeders.

An additional explanation for the persistence of an unreduced introgression may be related to the markers employed by breeding programs when conducting marker assisted selection (MAS). For much of the time since the *I-3* gene was introduced into cultivated germplasm, resources for marker development have been limited. Early breeding efforts relied on the *Got-2* isozyme marker for selection, which is located near 63.32 Mb on the SL2.50 tomato genome assembly (Bournival et al. 1989). Wang et al. (2007) subsequently developed the *Got-B* PCR-based marker from a bacterial artificial chromosome that contained the gene corresponding to the *Got-2* isozyme marker. The *I-3* gene was later mapped between markers CT226 and TG572 by Hemming et al. (2004), and Lim et al. (2006) reported the use of a CT226 CAPS marker in the breeding program in Queensland. Barillas et al. (2008) also described several *I-3* markers for use in breeding programs, including P7-43BF1 (designed from C2_At2g20830), PTG190 (designed from TG190), and P7-43D (designed from SGN-U321614). But whereas CT226 is located quite close to *I-3* at 63.49 Mb, the markers described by Barillas et al. (2008) are located at or upstream of 62.77 Mb. Thus, prior to the fine-mapping of *I-3* by Lim et al. (2008), the majority of the *I-3*-linked markers reported in the literature were located at or above 63.49 Mb. In this study, we observed very little recombination above

this region. In light of this, and together with our finding that the bacterial spot sensitivity locus was mapped somewhere above marker *7g501* (63.18 Mb), it is possible that markers located above 63.49 Mb are more tightly linked with the bacterial spot sensitivity locus than with *I-3*. Thus, to the extent that commercial and private breeding programs have relied on such markers in their MAS programs, selection efforts may have unintentionally been biased toward retaining the bacterial spot sensitivity locus, while selecting against many of the useful recombination events occurring between this locus and the *I-3* gene.

A logical product of this research will be the development and deployment of improved *Fol3* germplasm that contains a reduced *I-3* introgression and is potentially devoid of any negative effects toward horticultural performance. Although several of our *I-3* RILs should be free of the association with bacterial spot sensitivity and may be useful for this, it will be strategically important to advance the smallest possible introgression, since it is not known whether any other negative effects associated (e.g., reduced fruit size) may be due to still more tightly linked alleles than we have found here. To develop a minimal introgression, we employed a strategy similar to that described by Lim et al. (2006), wherein recombinants with opposing introgressions are crossed, and a product of homologous crossing-over is selected in the F_2. This approach was successfully used in the development of Fla. 8923, which contains a minimal *Ty-3* introgression (Hutton et al. 2015). The most suitable RILs for accomplishing this with *I-3* include either R18 or R26, crossed with either R12 or R15 (Fig. 1). In any of these cases, the resulting introgression size would be between 30 and 210 Kb. This material is currently under development and will be tested for presence or absence of other negative effects previously associated with *I-3*. Hutton et al. (2014) reported that current research efforts at UF/IFAS have focused on "non-blighting" (NB) resistance which achieved a high level of tolerance to bacterial spot in *I-3* breeding lines. Considering that these materials contain a typical, unreduced *I-3* introgression (Hutton, unpublished data), it may be possible to obtain an even higher level of bacterial spot tolerance in NB backgrounds by substituting the typical introgression with a minimal *I-3* introgression.

Author contribution statement JL and SFH each contributed to conception and design of experiments. JL, JC, NM, LM and SFH contributed to data acquisition and analysis.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval For this type of study formal consent is not required. This article does not contain any studies with human participants or animals performed by any of the authors.

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