Novel Sources of Resistance to *Fusarium oxysporum* f. sp. *lycopersici* Race 3 Among *Solanum pennellii* Accessions

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ABSTRACT. Fusarium wilt of tomato (*Solanum lycopersicum*), caused by fungal pathogen *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*), is one of the most important diseases in tomato production. Three races of the pathogen are described, and race-specific resistance genes have been applied in commercial tomato cultivars for controlling the disease. Race 3 (*Fol3*) threatens tomato production in many regions around the world, and novel resistance resources could expand the diversity and durability of *Fol* resistance. The wild tomato species, *Solanum pennellii*, is reported to harbor broad resistance to *Fol* and was the source of two known *Fol3* resistance genes. In this study, we evaluated 42 *S. pennellii* accessions for resistance to each fusarium wilt race. F₁ plants, developed from crossing each accession with the *Fol3* susceptible line ‘Suncoast’, were evaluated for *Fol3* resistance, and BC₁F₁ plants were screened to determine the likelihood that *Fol3* resistance was based on a novel locus (*loci*). Nearly all accessions showed resistance to *Fol3*, and many accessions were resistant to all races. Evaluation of F₁ plants indicated a dominant resistance effect to *Fol3* from most accessions. Genetic analysis indicated 24 accessions are expected to contain one or more novel *Fol3* resistance loci other than an allele near the *I-3* locus. To investigate genetic structure of the *S. pennellii* accessions used in this study, we genotyped all 42 accessions using genotyping by sequencing. Approximately 20% of the single nucleotide polymorphism (SNP) loci were heterozygous across accessions, likely due to the outcrossing nature of the species. Genetic structure analysis at 49,120 unique SNP loci across accessions identified small but obvious genetic differentiations.

Fusarium wilt, caused by the fungal pathogen *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*), is a serious disease of tomato (*Solanum lycopersicum*) grown in warm production regions. The pathogen penetrates the root and infects the vascular system, blocking the supply of water and nutrition, and results in wilting, stunting, and, ultimately, plant death. The disease can cause devastating crop losses, and heavily infested fields were often abandoned before the availability of resistant cultivars (McGrath et al., 1987; Scott and Jones, 1989). Cultural practices, such as raising the soil pH level, using nitrate-nitrogen, and exercising sanitary

practices to reduce spread of inoculum, can contribute to disease management, especially when combined with soil fumigation (Jones and Overman, 1985; Jones and Woltz, 1968, 1983). However, host resistance is the most effective and economical means of controlling *Fol* and has been the primary strategy for controlling *Fol* in field-grown tomato production since the 1940s.

There are three races of *Fol* that infect tomato, and these are distinguished based on their response to three dominant, race-specific resistance genes, each derived from wild tomato species. The *I* (immunity to fusarium wilt) and *I-2* genes confer resistance to races 1 (*Fol1*) and 2 (*Fol2*), respectively. Both genes are located on chromosome 11 and were introgressed from accessions of *S. pimpinellifolium* (Bohn and Tucker, 1939; Paddock, 1950; Simons et al., 1998; Stall and Walter, 1965). *I-3*, which was introgressed from *S. pennellii* accession LA716, is located on chromosome 7 and confers resistance to race 3 (*Fol3*)

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and is reported to confer resistance to races 1 and 2 as well (Lim et al., 2008; Scott and Jones, 1989). Currently, only the *I*, *I-2*, and *I-3* genes are used for *Fol* resistance in commercial cultivars; but besides these, several other resistance genes have been reported. Sela-Buurlage et al. (2001) described *I-4*, *I-5*, and *I-6* in *S. lycopersicum* and *S. pennellii*, each conferring *Fol2* resistance. More recently, Gonzalez-Cendales et al. (2016) cloned a second dominant *Fol3* resistance gene, *I-7*, from *S. pennellii* accession LA1724 (PI 414773). Notably, these authors found that the *I-7* transgene conferred resistance against all three races of *Fol*, suggesting that *I-7* may provide broad efficacy. However, expression of this transgene was driven by the CaMV 35S promoter, and the effect of *I-7* against other races when under its native promoter has not been clearly demonstrated. Currently, *I-7* is not widely used for the development of commercial cultivars, likely because *I-3* is already deployed and provides effective *Fol3* control, and also because it was only relatively recently that *I-7* was distinguished from *I-3* (Lim et al., 2006).

Fol3 was originally discovered in Australia in 1978 and in Florida in 1982, and it now exists in many production regions around the world (Cai et al., 2003; Correll and Jones, 2014; Grattidge and O'Brien, 1982; Volin and Jones, 1982). Cultivars containing *I-3* have been commercially available since the early 1990s, but there has been considerable difficulty developing commercially acceptable *Fol3*-resistant hybrids due to the association of *I-3* with several negative traits. Some of the earliest *Fol3*-resistant parental lines showed a higher incidence of blossom end rot than susceptible lines, and this issue is occasionally observed today (Scott 2004). It was also reported that *I-3* has a negative effect on fruit size, resulting in a lower percentage of extra-large fruit—the size for which growers usually receive higher prices (Scott, 1999). Furthermore, the *I-3* locus has been associated with greater sensitivity to bacterial spot disease caused by multiple *Xanthomonas* species, and Hutton et al. (2014) demonstrated that homozygosity for *I-3* resulted in up to 20% more disease severity compared with homozygosity for the susceptible allele. Fortunately, the effects on fruit size and bacterial spot are caused by linkage drag, and reducing the size of the *I-3* introgression can eliminate these problems while maintaining *Fol3* resistance (Chitwood-Brown et al., 2021; Li et al., 2018).

Such negative effects are not associated with the *I-7* introgression (Chitwood-Brown et al., 2021) and neither have they been reported for the *I* or *I-2* introgressions, which are common resistance genes in much of the commercial germplasm worldwide. In light of this, we hypothesize that problems such as these may be specific to the *I-3* introgression, and that alternative, novel *Fol3* resistance genes might be free of such effects. Furthermore, the discovery and deployment of novel resistance genes may play a critical role in preventing the emergence of new *Fol* races in the future by expanding the diversity of *Fol3* resistance mechanisms. There are more than 40 *S. pennellii* accessions available from the C.M. Rick Tomato Genetics Resource Center (TGRC) at the University of California, Davis (TGRC, 2011). Scott and Jones (1990) tested many of these and reported broad resistance to multiple *Fusarium* species in all accessions tested. It is possible that *Fol3* resistance in some accessions is based on genetics other than *I-3* and *I-7*. Considering this, a genetic structure analysis may provide insight into the relationship among these accessions, allowing research efforts to be focused on a smaller number of accessions that are genetically less similar to one another and possibly to retain diversity

in resistance alleles. Likewise, such information may be useful for other research efforts seeking to mine *S. pennellii* for specific traits or novel alleles.

In this study, we investigate a large collection of *S. pennellii* accessions as potential sources of novel *Fol3* resistance. We characterize 42 accessions for resistance to all three races of fusarium wilt and identify potential sources of broadly effective resistance alleles. We also evaluate interspecific F₁ and backcross populations corresponding to each accession to identify those that are likely sources of novel, *Fol3* resistance loci. Finally, we use genotyping by sequencing [GBS (Elshire et al., 2011)] to generate a collection of SNPs between *S. pennellii* accessions and the tomato reference genome for genetic structure analyses.

Materials and Methods

PLANT MATERIALS. Seeds of 42 *S. pennellii* accessions were obtained from TGRC. Each accession was increased by manually sib-mating at least six plants of each accession in a greenhouse located at the University of Florida Gulf Coast Research and Education Center (GCREC) in Wimauma, FL. Information regarding geographic origin for each accession was obtained from the TGRC online database, including latitude, longitude, elevation, and habitat of the collection site, and collection sites were pinpointed on a map (Fig. 1). For each accession, an interspecific F₁ was made by bulking pollen from six or more plants of the accession and hybridizing with the *Fol3* susceptible cultivar, Suncoast (Scott et al., 1985). In the F₁, pollen was likewise bulked from six or more *Fol3*-resistant plants and then backcrossed to 'Suncoast' to create modified BC₁F₁ populations for each accession.

Similarly, resistance from accessions LA750, LA1297, LA1299, LA1367, LA1522, LA1734, and LA1926 was advanced into cultivated tomato to the fourth backcross using a modified backcrossing approach, wherein disease assays were again used at each generation to identify resistant plants for backcrossing. Molecular markers linked to *I-3* and *I-7* (Supplemental Table 1) were used to select away from these known loci and advance novel *Fol3* resistance. We selected against the *I-3* locus at the BC₁F₁ generation for most accessions (except for LA1926, for which the *I-3* locus was maintained); and we tested to confirm absence of the *I-7* locus in BC₄F₁ plants. Screening for *I-7* was not accomplished sooner because these generations were advanced before the discovery of the chromosomal location of *I-7* and the development of genetic resources for this locus by Gonzalez-Cendales et al. (2016). Seed was collected from individual BC₄F₁ plants for each population resulting in segregating BC₄F₂ populations.

Throughout all experiments, seed of accessions, interspecific F₁s, and BC₁F₁ populations were pretreated in a 2.7% sodium hypochlorite solution for 30 min before sowing in spent coal medium (Black Beauty; Harsco Minerals International, Mechanicsburg, PA) in a growth room. Controls used for disease screens included 'Bonny Best' (susceptible to all races), 'Manapal' [Walter et al., 1961 (contains *I*; resistant to *Fol1* but susceptible to *Fol2* and *Fol3*)], 'Horizon' [Scott et al., 1985 (contains *I* and *I-2*; resistant to *Fol1* and *Fol2* but susceptible to *Fol3*)], and Fla. 7547 [Scott and Jones, 1995 (contains *I*, *I-2*, and *I-3*; resistant to *Fol1*, *Fol2*, and *Fol3*)].

Fig. 1. Genetic structure of 42 *Solanum pennellii* accessions used in this study. (A) Genetic structure of 42 *S. pennellii* accessions primarily corresponds with geographic areas. Symbols (circle, empty triangle, cross) are based on three genetic clusters inferred using discriminant analysis of principal components (DAPC) shown on Supplemental Fig. 1. A single or double asterisk indicates the presence of single or multiple novel *Fol3* resistance loci, respectively. (B) Neighbor-joining tree of 42 *S. pennellii* accessions also reveals an association between accessions' geographic origins and phylogenetic clustering. Accessions in the tree are represented by different symbols: empty square (accessions collected north of latitude 10°S); half-filled square (between latitudes 10°S and 14°S); and filled square (south of latitude 14°S).

FUSARIUM WILT DISEASE SCREENING. Fungal strains of *Fol* races 1 (GEV2997), 2 (GEV3520), and 3 (GEV1400) were obtained from the University of Florida Plant Pathology laboratory at GCREC. The pathogen was grown on potato dextrose agar media at 28 °C for 1 week before inoculation. Inoculum was prepared by removing the fungal colony with agar from plates and blending into 100-mL deionized water per plate, then adjusting the fungal suspension to a concentration of 10^7 spores/mL. For each race inoculation, 10- to 14-d-old seedlings were inoculated by dipping the roots into inoculum for 15 s. Inoculated seedlings were immediately transplanted into 128-well trays (38 cm³ cell size; Speedling, Sun City, FL) filled with peat-based soilless media (Speedling) and placed in the greenhouse for disease development. Four to eight plants each of a resistant and a susceptible control were included in each tray; controls included 'Bonny Best' and 'Manapal' for *Fol1* screens, 'Manapal' and 'Horizon' for *Fol2* screens, and 'Horizon' and Fla. 7547 for *Fol3* screens. Plants were evaluated for disease incidence $\approx$ 3 weeks after inoculation according to procedures described previously (Scott et al., 2004). Briefly, plants were rated as diseased if they showed any characteristic external symptoms of fusarium wilt, such as stunting, yellowing of foliage, wilting, enlarged hypocotyl, or death. Stems of plants with questionable symptoms were dissected and rated as diseased if they had vascular browning. Otherwise, plants were scored as healthy and were considered resistant, and numbers of healthy and diseased plants were recorded.

Three separate disease screens were used to evaluate resistance of *S. pennellii* accessions to *Fol1*, *Fol2*, and *Fol3*. Interspecific F_1 s were evaluated in two separate *Fol3* screens, and both BC_1F_1 and BC_4F_2 populations were evaluated in single *Fol3* screens. The two screens of interspecific F_1 s produced similar results, so plant numbers were combined across them. As

sufficient plants were available, 32 plants of each accession and each F_1 , 80 plants of each BC_1F_1 , and 56 plants of each BC_4F_2 were evaluated in each disease screen.

GENETIC ANALYSIS OF RESISTANCE TO *FOL3*. Each accession and its interspecific F_1 were evaluated for *Fol3* resistance to assess the presence/absence of dominant *Fol3* resistance alleles. BC_1F_1 families were subsequently evaluated to determine the likelihood that *Fol3* resistance is controlled completely or in part by the *I-3* locus, or by a locus/loci besides *I-3*. Three molecular markers, linked to the *I-3* locus and distinguishing *S. pennellii* and *S. lycopersicum* alleles, were used for determining the presence of an *I-3* locus effect. Before disease development, BC_1F_1 plants were individually screened with one or more of these markers to determine genotype at the *I-3* locus. For each BC_1F_1 backcross population, each plant was assigned to one of the following four groups: 1) healthy and heterozygous for an introgression at the *I-3* locus, 2) healthy and lacking this introgression, 3) diseased and heterozygous for this introgression, or 4) diseased and lacking this introgression.

Within each BC_1F_1 family, a χ^2 contingency test was used to determine if the presence of an *S. pennellii* introgression at the *I-3* locus affected *Fol3* resistance. The null hypothesis for this test was that an introgression at this locus had no effect on resistance, and there would therefore be no difference in the healthy to diseased ratio for plants that possessed and for plants that lacked an *I-3* locus introgression. When the null hypothesis was rejected, it was concluded that resistance in the accession is conferred completely or in part by the *I-3* locus. Then, where the null hypothesis was accepted, it was concluded that resistance in the accession is not based on *I-3* but is based entirely on alleles other than *I-3*.

A χ^2 test was also used to test for the presence of novel resistance loci. For families of accessions determined to have no

effect at the *I-3* locus, calculations were based on all BC₁F₁ plants; and for families of accessions that were determined to harbor a resistance allele at/near the *I-3* locus, calculations were based only on plants lacking an *S. pennellii* introgression in this region. Numbers of healthy and diseased plants were tested for goodness of fit to an expected 1:1 and/or 3:1 ratio to test for the presence of one or two alternative resistance loci, respectively. Where families had an unacceptable fit to the 3:1 ratio due to an excess of healthy plants, it was concluded that resistance may be conferred by more than two alternative resistance loci.

Fol3 resistance was further evaluated in BC₄F₂ families, and molecular markers linked to *I-3* were used as previously described to confirm the presence or absence of this locus. Chi-square analyses of BC₄F₂ families were used to test healthy to diseased ratios for goodness of fit to an expected 3:1 or 15:1 ratio for the presence of one or two resistance genes, respectively.

GENOTYPING BY SEQUENCING. Young foliar tissue was collected separately from two individual plants from each *S. pennellii* accession. Genomic DNA was extracted using the Plant DNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA was normalized to 5 ng·μL⁻¹ based on quantification with a multimode microplate reader (Synergy 2; Biotek Instruments, Winooki, VT) with the Quant-iT PicoGreen (Thermo Fisher Scientific, Waltham, MA) double-stranded DNA. An *ApeKI*-based GBS library was constructed using previously published protocols, barcode-adapters, and polymerase chain reaction primers (Elshire et al., 2011). The final GBS library (total 84 libraries; two libraries per each accession) was analyzed by automated electrophoresis (2100 Bioanalyzer; Agilent Technologies, Santa Clara, CA) before sequencing to confirm and examine fragment size distribution and quantities. The GBS library was submitted for 101-cycle single-end sequencing on one lane of an Illumina HiSeq 2500 instrument (Illumina, San Diego, CA) at the Weill Cornell Medical College Genomics Resources Core Facility.

One multiplexed sequencing run generated a total of 140,179,147 reads with barcodes from 42 *S. pennellii* accessions. The raw sequencing data were processed using the TASSEL 5 GBS v2 pipeline (Bradbury et al., 2007). Bowtie 2 with the –very-sensitive option (Langmead and Salzberg, 2012) was used to align the reads to the tomato reference genome (Tomato SL2.50 ITAG2.4; Sol Genomics Network, 2019). Only 80–base pair kmers (reads) present at least five times in the dataset were considered. Minimum quality score within the barcode and read length was set for 20. SNPs were not filtered by minor allele frequency. For each SNP, only sequence polymorphism common to both individual plant samples of each accession were considered. SNPs with >95% missing data were discarded.

GENETIC STRUCTURE ANALYSES. We explored genetic differentiation among 42 *S. pennellii* accessions genotyped using the GBS method. We used K-means clustering, which clusters objects to maximize between group variation, for K = 1 through 8 implemented in the adegenet package (Jombart, 2008) for the R software (version 3.0.1; R Foundation, Vienna, Austria). We used the Bayesian information criterion to select the number of clusters that best describes those data. Using the multivariate method [discriminant analysis of principal components (DAPC) (Jombart et al., 2010)], accessions were grouped into three clusters that were genetically diverse from one another. In addition, we built a phylogenetic tree for these accessions using the

neighbor-joining method implemented in TASSEL 5 and the same genotype dataset that was used for DAPC.

Results

FOL RESISTANCE AMONG *S. PENNELLII* ACCESSIONS. Disease screens of 42 accessions confirmed high levels of resistance to *Fol* in the *S. pennellii* species (Table 1). Overall, these accessions were less resistant to *Fol1* than to *Fol2* or *Fol3*. Whereas 27 accessions demonstrated some level of *Fol1* resistance (≥50% healthy plants), only five of these exceeded 90% healthy plants, whereas 14 accessions had less than 50% healthy plants. Among the latter, accessions LA1926 (6% healthy) and LA2657 (15% healthy) were considered susceptible to *Fol1*. Most accessions were resistant to *Fol2*, and the percentage of *Fol2*-resistant plants ranged from 50% to 90% among these. LA1926 was the only accession clearly susceptible to *Fol2*, having 7% healthy plants. Resistance was highest and most consistent for *Fol3*, for which 31 accessions had more than 90% healthy plants, and 39 accessions had more than 70% healthy plants. LA1733 and LA1912 had less than 70% healthy plants.

Many of the interspecific F₁s were resistant to *Fol3* and displayed similar levels of resistance as the parental accession, suggesting dominant gene action and/or homogeneity of resistance alleles within the accession. Some accessions (LA1724, LA1732, LA1809, LA1911, LA1912, LA1926) displayed lower levels of resistance in the interspecific F₁, suggesting a lack of dominance and/or segregation of resistance. The possibility of heterogeneity for resistance was suggested for LA1912, as this parental accession LA1912 had only 63% healthy plants in the *Fol3* assay and 27% healthy plants in the interspecific F₁ (Table 1).

To further characterize *Fol3* resistance in these materials, BC₁F₁ families corresponding to 40 of the accessions were evaluated in *Fol3* disease screens, and molecular markers were used to detect the presence of an introgression at the *I-3* locus (Table 2). Because only two crosses to susceptible cultivated tomato had been made, segregation for an introgression at the *I-3* locus was expected for all families. However, this locus may or may not confer *Fol3* resistance. To identify potential sources of novel resistance loci, we sought first to determine whether the *I-3* introgression contributed to *Fol3* resistance; and second, whether additional resistance loci are likely to exist.

Segregation for *Fol3* resistance was observed for each BC₁F₁ family. An introgression near the *I-3* locus was detected in all populations except for that derived from LA751; here, the linked markers were not polymorphic, and we were therefore unable to test for an *I-3* locus effect. Chi-square analysis was used to determine an effect of the *I-3* locus by comparing the total numbers of healthy and diseased plants with and without an introgression at *I-3*, which indicated the presence of a resistance allele at or near this locus in 18 of the populations (Table 2). For these populations, the presence of alternative resistance loci was determined with χ² tests using the plants without an introgression at *I-3*. As expected, an effect at *I-3* was detected in LA716, the source of *I-3* in commercial germplasm, but analysis also indicated the presence of an alternative resistance locus besides *I-3* in this accession. The possibility of one additional resistance locus was also indicated for LA750, LA1272, LA1297, LA1299, LA1340, LA1515, LA1522, LA1649, and LA1942. In LA1273, LA1303, LA1735, LA1911, LA1920, LA1926, and LA1946, segregation ratios among plants lacking an introgression at the

Table 1. Resistance among *Solanum pennellii* accessions to *Fusarium oxysporum* f. sp. *lycopersici* (Fol) races 1, 2, and 3, and resistance of corresponding *Solanum lycopersicum* × *S. pennellii* interspecific hybrids to race 3.

Accession ^z	Race 1			Race 2			Race 3			Interspecific hybrids ^y		
	Healthy (no.)	Diseased (no.)	Healthy (%)	Healthy (no.)	Diseased (no.)	Healthy (%)	Healthy (no.)	Diseased (no.)	Healthy (%)	Healthy (no.)	Diseased (no.)	Healthy (%)
LA716	29	3	91	32	0	100	31	0	100	44	0	100
LA750	14	21	40	25	23	52	32	0	100	48	0	95
LA751	8	24	25	24	8	75	32	0	100	61	3	95
LA1272	24	8	75	31	1	97	32	0	100	56	3	95
LA1273	24	8	75	24	8	75	31	1	97	52	11	83
LA1275	25	7	78	20	8	71	30	2	94	51	13	80
LA1277	27	5	84	31	1	97	31	1	97	58	0	100
LA1282	15	17	47	25	3	89	23	9	72	61	1	98
LA1297	26	6	81	25	7	78	31	1	97	53	9	86
LA1299	28	4	88	27	1	96	32	0	100	59	1	98
LA1302	32	0	100	29	3	91	32	0	100	54	0	100
LA1303	21	11	66	17	7	71	31	1	97	51	13	80
LA1340	15	17	47	25	7	78	31	1	97	16	0	100
LA1356	23	9	72	24	4	86	32	0	100	52	3	95
LA1367	13	19	41	22	10	69	32	0	100	51	13	80
LA1376	12	20	38	20	8	71	32	0	100	53	3	95
LA1515	22	10	69	24	8	78	32	0	100	51	13	80
LA1522	20	12	63	30	2	94	32	0	100	64	0	100
LA1649	3	1	75	4	2	67	21	0	100	59	5	92
LA1656	30	2	94	29	3	91	31	1	97	63	1	98
LA1657	29	3	91	32	0	100	30	2	94	37	1	97
LA1674	25	7	78	31	1	97	32	0	100	54	2	96
LA1693	10	12	46	18	8	69	28	0	100	60	4	94
LA1724	31	9	78	19	4	83	32	0	100	24	40	38
LA1732	33	7	83	21	3	88	32	4	89	29	35	45
LA1733	—	—	—	—	—	—	2	1	67	57	3	95
LA1734	22	10	69	28	4	88	31	1	97	63	1	98
LA1735	11	3	73	8	2	80	22	6	79	40	13	76
LA1809	17	47	27	40	24	63	32	0	100	38	26	59
LA1911	5	16	24	15	18	46	29	3	91	10	54	16
LA1912	17	15	53	26	6	81	20	12	63	17	47	27
LA1920	7	17	29	18	5	78	20	7	74	47	17	73
LA1926	1	15	6	1	14	7	29	3	91	33	20	62
LA1940	28	4	88	32	0	100	32	0	100	62	2	97
LA1941	25	7	78	29	3	91	31	1	97	58	6	91
LA1942	32	0	100	24	0	100	28	10	74	54	10	84
LA1943	32	10	76	51	1	98	32	0	100	63	1	98
LA1946	28	5	85	27	3	90	27	5	84	18	0	100

(Continued on next page)

Table 1. (Continued)

Accession ^z	Race 1			Race 2			Race 3			Interspecific hybrids ^y		
	Race 1			Race 2			Race 3			Interspecific hybrids ^y		
	Healthy (no.)	Diseased (no.)	Healthy (%)	Healthy (no.)	Diseased (no.)	Healthy (%)	Healthy (no.)	Diseased (no.)	Healthy (%)	Healthy (no.)	Diseased (no.)	Healthy (%)
LA2560	22	23	49	22	5	82	25	7	78	5	0	100
LA2580	28	4	88	21	1	96	27	5	84	48	7	87
LA2657	7	41	15	14	4	83	28	4	86	46	16	72
LA2963	29	43	40	46	10	82	29	3	91	63	1	98
Fla. 7547	—	—	—	—	—	—	48	0	100	192	0	100
Horizon	—	—	—	56	0	100	1	47	2	18	174	9
Manapal	68	0	100	5	53	9	—	—	—	—	—	—
Bonny Best	0	68	0	—	—	—	—	—	—	—	—	—

^zControls were Fla. 7547 (resistant to *FolI-3*), 'Horizon' (resistant to *FolI-2*), 'Manapal' (resistant to *FolI*), and 'Bonny Best' (susceptible to all races).

^yInterspecific hybrids were developed by crossing the *FolI*-susceptible cultivar 'Suncoast' with *FolI*-resistant plants of each *S. pennellii* accession.

I-3 locus did not support the presence of additional resistance alleles, and these accessions may thus serve little utility toward the identification of novel *Fol3* resistance alleles. In LA1302, segregation ratios suggested the possibility of two resistance loci other than *I-3*.

For the 21 populations demonstrating no effect at the *I-3* locus, the potential number of novel resistance loci were predicted with χ^2 tests using the total number of plants screened (Table 2). Based on these analyses, resistance in 10 of the BC₁F₁ families may be conferred by a single locus, while two resistance loci likely confer *Fol3* resistance in accessions LA1282, LA1656, and LA1674.

Besides those mentioned previously, eight BC₁F₁ families fit none of the segregation ratios tested, primarily because of a deficiency of resistant plants (Table 2). LA1912 was among these and demonstrated the lowest levels of resistance of all BC₁F₁ families. LA1367 was also among these, but in the case of this accession, there was no deficiency of resistant plants. Rather, its 51:29 ratio of healthy:diseased plants was slightly skewed such that it fit neither a 1:1 nor a 3:1 segregation ratio. Thus, although χ^2 analyses were not conclusive, there is evidence that LA1367 contains at least one alternative *Fol3* resistance locus.

We continued to advance resistance from some accessions into cultivated tomato by selecting resistant plants from disease assays for backcrossing. Resistance to *Fol3* in several BC₄F₂ families is shown in Table 3. Chi-square tests of segregation ratios were used to substantiate predictions made in the BC₁F₁ families. We also used molecular markers linked to *I-3* to confirm absence of an introgression (or in the case of LA1926, genotype for each BC₄F₂ plant) at this locus in each family. LA750, LA1297, LA1299, and LA1522 were previously determined to have an effect at the *I-3* locus in addition to one alternative resistance locus. Here, *I-3*-linked markers confirmed the absence of an *I-3* introgression in these BC₄F₂ families, and segregation ratios further supported the presence of one alternative locus conferring resistance in each family. As mentioned previously, the presence of at least one alternative *Fol3* resistance locus was evidenced, although segregation ratios in its BC₁F₁ family did not fit either a 1:1 or 3:1 ratio. However, χ^2 analyses of the BC₄F₂ population for LA1367 indicated two alternative genes conferring resistance (Table 3). LA1734 was formerly determined to have no effect at the *I-3* locus and to contain one alternative resistance locus; analyses confirmed the absence of any introgression at the *I-3* locus and supported the presence of a single novel locus conferring resistance. Resistance from LA1926, which was previously determined to be conferred solely by the *I-3* locus, was also advanced, and the segregating BC₄F₂ population was subjected to disease and marker screens, as previously described. Chi-square analyses confirmed segregation of a single resistance locus, and further testing verified that the *I-3* locus in fact cosegregated with resistance in this line ($P < 0.0001$).

GENETIC STRUCTURE ANALYSES FOR *S. PENNELLII* ACCESSIONS.

In this study, all accessions originated from Peru (Fig. 1A). Their geographic collection sites are distributed along the entire west coast, with the greatest distance between collection sites being 1537 km between accessions LA1089 and LA2963. The smallest distance between collection sites was less than 1 km, and this was observed between LA751 and LA1282, between LA1911 and LA1912, and between LA1942 and LA1943.

Table 2. Genetic analysis of *Solanum lycopersicum* × *Solanum pennellii* BC₁F₁ populations for identifying potential sources of novel resistance to fusarium wilt race 3.

BC ₁ F ₁ population ^z	<i>I-3</i> locus introgression					Alternative resistance loci			
	Heterozygous		Absent		Locus effect		χ^2 probability ^x		Loci (no.)
	Healthy (no.)	Diseased (no.)	Healthy (no.)	Diseased (no.)	χ^2 probability	Conclusion ^y	(1:1)	(3:1)	
LA716	37	4	17	20	0.000	Yes	0.622	0.000	1
LA750	32	5	14	24	0.000	Yes	0.105	0.000	1
LA751	0	0	20	40	—	—	0.010	0.000	0
LA1272	39	5	11	18	0.000	Yes	0.194	0.000	1
LA1273	22	14	6	16	0.012	Yes	0.033	0.000	0
LA1275	14	19	20	26	0.926	No	0.216	0.000	1
LA1277	21	17	10	18	0.116	No	0.622	0.000	1
LA1282	35	14	16	13	0.145	No	0.007	0.050	2
LA1297	28	11	14	23	0.003	Yes	0.139	0.000	1
LA1299	20	0	10	10	0.000	Yes	1.000	0.010	1
LA1302	15	0	11	4	0.032	Yes	0.071	0.881	2
LA1303	15	11	11	28	0.017	Yes	0.006	0.000	0
LA1340	23	13	16	23	0.048	Yes	0.262	0.000	1
LA1356	30	17	12	16	0.077	No	0.299	0.000	1
LA1367	11	3	40	26	0.204	No	0.014	0.020	— ^w
LA1376	20	11	19	17	0.331	No	0.179	0.002	1
LA1515	22	5	27	26	0.008	Yes	0.891	0.000	1
LA1522	27	9	16	28	0.001	Yes	0.070	0.000	1
LA1649	38	9	12	18	0.000	Yes	0.273	0.000	1
LA1656	22	11	18	10	0.845	No	0.015	0.089	2
LA1674	34	14	19	13	0.288	No	0.004	0.071	2
LA1693	13	8	16	21	0.172	No	1.000	0.000	1
LA1724	11	15	20	19	0.478	No	0.710	0.000	1
LA1732	9	10	12	18	0.612	No	0.317	0.000	1
LA1733	10	16	11	26	0.469	No	0.008	0.000	0
LA1734	7	6	11	16	0.435	No	0.527	0.000	1
LA1735	9	14	9	43	0.041	Yes	0.000	0.000	0
LA1809	10	17	10	32	0.237	No	0.000	0.000	0
LA1911	7	6	6	22	0.038	Yes	0.002	0.000	0
LA1912	3	34	2	29	0.794	No	0.000	0.000	0
LA1920	20	17	8	34	0.001	Yes	0.000	0.000	0
LA1926	12	6	1	16	0.000	Yes	0.000	0.000	0
LA1940	15	20	11	35	0.070	No	0.001	0.000	0
LA1941	20	19	21	20	0.996	No	0.823	0.000	1
LA1942	25	9	21	20	0.048	Yes	0.876	0.000	1
LA1943	23	16	23	15	0.890	No	0.087	0.002	1
LA1946	10	10	8	33	0.014	Yes	0.000	0.000	0
LA2580	4	11	20	33	0.428	No	0.015	0.000	0
LA2657	13	17	9	32	0.054	No	0.001	0.000	0
LA2963	7	29	6	33	0.643	No	0.000	0.000	0

^zBackcross populations were developed from crossing fusarium wilt race 3-resistant plants from each *S. pennellii* accession and from each subsequent interspecific hybrid with the race 3-susceptible cultivar Suncoast.

^yAn effect at the *I-3* locus was determined using χ^2 contingency tests in which the null hypothesis was that no difference was observed in the ratio of healthy to diseased plants whether the *I-3* locus was present or absent. “No” indicates that the null was accepted and there was no effect of the *I-3* locus, and “yes” indicates that the null was rejected and the locus was determined to have an effect.

^xChi-square tests were conducted using the total number of plants when an *I-3* effect was absent in an BC₁F₁ population; and tests were conducted using only *i-3/i-3* plants when an *I-3* effect was present in an BC₁F₁ population ($P < 0.05$). The analysis tested whether segregation fit a 1:1 or 3:1 (healthy to diseased) ratio, indicating the presence of one or two resistance loci, respectively.

^wAlthough the number of healthy and diseased plants for the population with resistance derived from LA1367 fit neither a 1:1 nor a 3:1 segregation ratio, the number of healthy plants is evidence that at least one *Fol3* resistance locus is present.

Analysis of data from GBS identified 1,378,871 SNPs across all accessions (Supplemental Tables 2 and 3). The number of monoallelic SNPs per accession ranged from 24,157 to 33,735, and the number of heterozygous SNPs per accession

ranged from 3005 to 7205. The proportion of heterozygous SNPs was high, ranging from 10% to 27% across accessions and averaging 20%. This was likely due to the outcrossing nature of the species, and for this reason, heterozygous SNPs

Table 3. Analysis of advanced resistance to fusarium wilt race 3 in *Solanum lycopersicum* BC₄F₂ populations.

Resistance derived from	Healthy (no.)	Diseased (no.)	χ^2 probability ^z	
			(3:1)	(15:1)
LA750	45	11	0.355	0.000
LA1297	36	20	0.064	0.000
LA1299	36	20	0.064	0.000
LA1367	49	7	0.031	0.053
LA1522	43	13	0.758	0.000
LA1734	47	9	0.123	0.002
LA1926	42	10	0.337	0.000
LA750	45	11	0.355	0.000

^zChi-square analyses were conducted to compare the segregation ratios of healthy and diseased plants to the ratios expected when one (3:1) or two (15:1) resistance genes are present.

were not excluded from further analyses. Upon comparing all SNP loci among the accessions, a final set of 49,120 unique SNP loci were determined, and these were used for subsequent analyses.

K-means clustering indicated that different patterns of genetic structure could be used to describe the genotype data of all 42 accessions (Supplemental Fig. 1). The mode of $K = 3$ found significant structuring between accessions collected from areas above an approximate latitude 14°S (two clusters with 29 accessions) and accessions collected from below this latitude (a single cluster with 13 accessions) (Fig. 1A). A third cluster with three accessions (LA751, LA1732, LA1303) showed no clear geographic differentiation from the rest of the accessions collected from areas above an approximate latitude 14°S, but was reassigned to a different genetic structure.

We also investigated genetic structure using a phylogenetic analysis, and this likewise revealed an association between geographic origins and the occurrence of phylogenetic clustering (Fig. 1B). Importantly, we observed that the accessions with novel *Fol3* resistance alleles are not confined to a single clade but are present in various clades. Among all accessions, the smallest pairwise genetic distance was between accessions LA716 and LA1943, and the largest genetic distance was between LA1943 and LA2560.

Discussion

Much of the disease resistance used in hybrid tomato breeding is conferred by dominant resistance genes. The present study investigated a collection of 42 *S. pennellii* accessions for their potential to harbor novel *Fol3* resistance loci. The use of screenings at the F₁, BC₁F₁, and subsequent BCF₁ generations has likely biased our results toward detection of dominant resistance alleles that confer a high level of resistance. We used χ^2 analyses to test segregation ratios in backcross families to determine which accessions are likely to contain novel *Fol3* resistance loci, thereby providing some direction for programs that may wish to pursue research in this area. Although these estimates can be useful, they may not be completely accurate in all cases, because segregation ratios in wide crosses such as ours can deviate from normal (Rick, 1969). The estimates were also based on the assumption that all detectable resistance alleles were homogeneously present in the interspecific F₁. Although we used only *Fol3*-resistant F₁ plants to generate BC₁F₁ seed, we recognize

that *S. pennellii* is primarily an outcrossing species, and that this assumption may have been violated in some instances if multiple resistance alleles were present and segregating within an accession.

Our screening confirmed a very high level of *Fol3* resistance in most *S. pennellii* accessions. Resistance to *Fol3* appeared to be dominant in most accessions, as evidenced by the high survival rates among interspecific F₁s. This is of particular practical importance, given the utility of dominant resistance genes for hybrid breeding. In general, survival rates in the F₁s were slightly lower relative to the donor accessions, which might indicate some level of incomplete dominance. But another likely explanation is that this resulted from heterogeneity of resistance, which may also explain why *Fol3* resistance deviated from 100% for several of the accessions. F₁ survival rates for some of the accessions (i.e., LA1724, LA1732, LA1911, and LA1912) were markedly low, possibly due to heterogeneity and/or heterozygosity of resistance within the accessions, incomplete dominance of resistance, or segregation distortion for the resistance alleles. In the analysis of BC₁F₁ populations, both LA1724 and LA1732 were predicted to contain one resistance locus. LA1724 is the reported source of *Fol3* resistance gene *I-7*, and it is likely that the resistance in this population is due to this gene. Furthermore, Catanzariti et al. (2015) demonstrated that *I-7* confers resistance to *Fol1* and *Fol2*, and we also observed LA1724 to have resistance to *Fol1* (78% healthy) and *Fol2* (83% healthy) (Table 1). Therefore, for the purpose of identifying novel sources of *Fol* resistance, this accession may be of minimal interest.

Several accessions displayed lower resistance or were susceptible to *Fol1* and/or *Fol2*. These accessions may therefore be less desirable as potential sources of novel alleles, if the goal is to identify sources of broad resistance toward improved durability. Bournival et al. (1990) suggested that the *I-3* introgression confers resistance to *Fol3* as well as *Fol1* and *Fol2*, but it was unclear from this work whether resistances to races 1 and 2 were conferred by *I-3* or by linked genes. Although Scott et al. (2004) and Sarfatti et al. (1991) reported that *Fol1* resistance in LA716 is conferred by the *I-1* gene located near *I-3*, findings of Do et al. (2016) rather suggest that *I-3* alone confers at least partial resistance to *Fol1* in addition to resistance to races 2 and 3. Despite the lack of continuity among these studies, the evidence that *I-3* provides at least partial resistance to multiple races supports that *I-3* should be pyramided with, rather than replaced by, any novel *Fol3* resistance alleles that may later be discovered, especially if these do not provide comparable disease control to *Fol* races 1 to 3 as *I-3*. Our findings demonstrate that resistance to all three *Fol* races is present in multiple accessions, but further work will be needed to determine if a specific novel *Fol3* resistance locus is likewise effective against races 1 and 2.

Whereas *Fol3* resistance cosegregated with the *I-3* locus in some backcross populations, 23 accessions are expected to contain one or more alternative resistance loci. Seven of these (i.e., LA750, LA1282, LA1340, LA1376, LA1522, LA1649, and LA1693) had questionable *Fol1* and/or *Fol2* resistance and thus may not be first choices in pursuit of novel, durable *Fol3* resistance genes. The remaining 16 accessions (i.e., LA716, LA1272, LA1275, LA1277, LA1297, LA1299, LA1302, LA1356, LA1515, LA1656, LA1674, LA1732, LA1734, LA1941, LA1942, and LA1943) are all strong candidate sources for novel *Fol3* resistance genes and also demonstrated high levels of *Fol1* and *Fol2* resistance. For each of these accessions, *Fol3* resistant BC₁F₁ plants that

lacked an *I-3* locus introgression were used for further backcrossing, and resistance was advanced to the fourth backcross. Segregation in BC₄F₂ families for several of these sources also supported the alternative locus predictions in the BC₁F₁. One alternative resistance locus likely confers resistance in BC₄F₂ families of LA750, LA1297, LA1299, LA1522, and LA1734; while LA1367 could contribute two novel resistance genes.

Because of our intentional selection efforts against known *Fol3* resistance loci, it is not expected that any of the advanced backcross populations generated through this work will contain *I-3*, *I-7*, or alternative alleles at these loci. However, it is possible that other reported *Fol* resistance loci may be responsible for the resistance responses that were observed. Sela-Buurlage et al. (2001) identified *I-5* and *I-6* from *S. pennellii* accession LA716, each of which conferred resistance to *Fol2*. The authors also identified regions on chromosome 11 that were associated with resistances to races 1 and 2, concluding that these regions may contain *S. pennellii* alleles of the *I* and/or *I-2* genes from *S. pimpinellifolium*. Further research is under way to determine the locations of alternative resistance loci in several accessions and to verify whether resistance is conferred by novel or by previously reported loci. In their study, Sela-Buurlage et al. (2001) reported the locus syntenic with the *I* gene to confer almost complete resistance to *Fol2*. This suggests the possibility that some alternative alleles may confer resistance to multiple races of fusarium wilt, increasing their utility toward developing more durable resistance to this pathogen.

Data from GBS was successfully used to identify SNPs and to genotype all 42 *S. pennellii* accessions used in this study. This information may provide a valuable resource for researchers wishing to use the *S. pennellii* species in their programs; for example, sequence polymorphisms identified in diverse sources could be used to capture existing genetic diversity and develop molecular markers linked to resistance in each specific accession or to obtain marker saturation near the target gene(s) region in mapping populations.

In general, the genetic structure of *S. pennellii* elucidated from GBS data evidences the divergence of the southern accessions from the northern accessions, corresponding with geographic areas that span $\approx 10^\circ$ latitude. However, no clear geographic pattern was evident with regard to presence of alternative *Fol3* resistance loci. In addition, genetic structure did not primarily correspond to the observed alternative loci. The range in genetic distance among all accessions was relatively small, which resulted from the fact that all accessions belong to the same species. Nonetheless, the distinguishable genetic structure among accessions means that the GBS data can potentially be used to classify accessions based on their similarity with one another. Thus, whether investigating novel *Fol3* resistance loci or other *S. pennellii* traits, researchers may choose to work with one or more accessions from each of the two major genetic clusters (viz. the circle and the cross symbols in Fig. 1) to maximize the genetic diversity represented in their populations. Likewise, the highly significant correlation between genetic distance and location of origin that was observed suggests that one could accomplish the same goal by arbitrarily choosing accessions from the northern and southern geographical collection sites. With regard to *Fol3* resistance, our hypothesis is that accessions that are least related to one another may be less likely to contain the same alternative resistance gene to *Fol3*, and this also could be investigated with further research.

Despite the adoption of cultural practices that improve management of fusarium wilt in tomato, the utilization of *Fol*-resistant cultivars has been the primary strategy for disease control since 1939. Cultivar resistance is primarily based on three single, dominant resistance genes: *I*, *I-2*, and *I-3*. Considering the history of R genes in many crops, together with the fact that both *I* and *I-2* were overcome with time, it is almost certain that *I-3* will eventually be overcome by the emergence of a new *Fol* strain(s). However, the introduction of additional *Fol* resistance genes for pyramiding with those currently being used is likely to improve the durability of resistance. As an example, the *I-3* and *I-7* genes belong to different gene families and also differ in their mechanisms of resistance to *Fol3*, and these genes are already being pyramided in some breeding programs (Catanzariti et al., 2015; Gonzalez-Cendales et al., 2016; Lim et al., 2006). Similarly, sources of novel *Fol3* resistance loci identified through the present study and currently being pursued may help to expand the *Fol* resistance gene resources that are available toward this end.

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Supplemental Fig. 1. Genetic clusters inferred using discriminant analysis of principal components (DAPC). Clusters are shown by different colors and inertia ellipses, and dots represent individual *Solanum pennellii* accessions. The red, blue, and yellow groups represent the circle, empty triangle, and cross symbols in Fig. 1A, respectively. The inset indicates the eigenvalues of the analysis, which retains two principal components.

Supplemental Table 1. Molecular markers used to detect the *Solanum pennellii* introgressions containing either *I-3* or *I-7*.

Gene	Marker (5'–3')	Type ^z	Restriction enzyme	Fragment size (bp) ^y	Physical position ^x (Mb)
<i>I-3</i>	<i>7g728</i> F: CACAAGTGCCTAAACATGACC R: TAACCAAATTTATTGGCTTTTGA	SCAR	none	132 vs. 117	63.41
	<i>7g923</i> F: ATTGGTTAAGTGAGACGTGTG R: TAATGAATGCTGTGGCTAGGA	SCAR	none	125 vs. 112	63.60
	<i>SLG-1</i> F: CGTTCATAATCTGCTAAGTTTCTA R: GGTTTCTATTACTCCAGCATCCTC	CAPS	<i>RsaI</i>	296,170 vs. 466	63.63
<i>I-7</i>	<i>I-7A</i> F: TTTCTTATTGGAACCATC R: GACTAAGTTTTGTGAGGCTA	PCR-HRM	none	49	61.64
	<i>I-7B</i> F: GATGCGTCAAGCTACTGAGTTCAC R: TGCAGCTTTCTTATTGGAACCATC	PCR-HRM	none	155	61.64

^zMarker type included SCAR = sequence characterized amplified region; CAPS = cleaved amplified polymorphic sequence; and PCR-HRM = polymerase chain reaction, high-resolution melting marker.

^ySize of detected amplicons/fragments: *Solanum lycopersicum* vs. *S. pennellii*.

^xPhysical locations of the markers on chromosomes 7 and 8 for *I-3* and *I-7*, respectively, are based on the tomato genome assembly, version SL2.50 (<http://solgenomics.net/>); “Mb” represents mega base.

Supplemental Table 2. Sequence polymorphisms identified in 42 *Solanum pennellii* accessions using the genotyping by sequencing method.

Accession	Mono-allelic SNPs ^z	Heterozygous SNPs ^y	Heterozygosity (%)
LA1943	29082	3005	10
LA716	33735	3756	11
LA2963	32445	4030	12
LA1946	32312	4878	15
LA2680	26057	3998	15
LA1724	26915	4147	15
LA1912	28018	4470	16
LA1733	26401	4292	16
LA750	29788	5019	17
LA1732	26039	4454	17
LA1911	27608	4759	17
LA1920	29121	5085	17
LA1272	26602	4867	18
LA1942	28496	5227	18
LA1656	27952	5168	18
LA1340	27773	5182	19
LA1941	29920	5588	19
LA1522	28244	5366	19
LA1734	27143	5239	19
LA2560	26807	5218	19
LA1926	29588	5852	20
LA1297	27359	5497	20
LA1649	28608	5838	20
LA1275	25622	5282	21
LA1809	25876	5427	21
LA1282	27266	5936	22
LA1302	26471	5821	22
LA1735	25301	5667	22
LA1299	24157	5487	23
LA1693	28057	6451	23
LA2657	29175	6750	23
LA1657	25434	5912	23
LA1515	25313	5885	23
LA1367	25897	6022	23
LA1303	27058	6420	24
LA1273	24927	6053	24
LA1277	25012	6201	25
LA1356	25085	6327	25
LA1674	26713	6798	25
LA751	26671	6994	26
LA1376	24340	6526	27
LA1940	26384	7205	27

^zSNP = single nucleotide polymorphism.

^ySNP-containing positions that have a heterozygous sequence genotype.