Scientia Horticulturae 201 (2016) 346–354

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Conventional and molecular marker-assisted selection and pyramiding of genes for multiple disease resistance in tomato

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ARTICLE INFO

Article history:
Received 4 October 2015
Received in revised form 5 February 2016
Accepted 7 February 2016

Keywords:
Begomovirus
Late blight
Bacterial wilt
Disease resistance
Solanum lycopersicum

ABSTRACT

Tomato (*Solanum lycopersicum* L.) is widely grown in the tropics but production is subject to high losses from diseases. AVRDC—The World Vegetable Center initiated a program to develop fresh market tomato lines resistant to begomoviruses causing tomato yellow leaf curl disease, *Phytophthora infestans* causing late blight, *Ralstonia solanacearum* causing bacterial wilt, *Stemphyllium* spp. causing gray leaf spot, *Fusarium oxysporum* f. sp. *lycopersici* race 2, and *Tobacco mosaic virus*. This work provides greenhouse, field, molecular marker, and laboratory protocols used in the screening and selection process that were applied to segregating populations during generation advance over three years to develop five multiple disease resistant F_7 fresh market tomato lines. Resistance of the five lines to the abovementioned diseases was confirmed in subsequent evaluations. Average yields of the five lines exceeded 100 t/ha under optimal temperatures in a dry season trial, but yields were reduced in a second trial under higher temperatures and rainfall. Seed of three multiple disease resistant $F_{7:8}$ lines is available from AVRDC (http://avrdc.org/seed/improved-lines/); these lines have potential for release as inbred line cultivars, hybrid parental lines, or breeding stock.

1. Introduction

Tomato (*Solanum lycopersicum* L.) is a widely grown vegetable throughout the tropics and subtropics and is an important source of vitamins A and C. Production of high value fruit and vegetables such as tomato offer some smallholders the opportunity to change from subsistence to commercial farming and substantially increase their incomes (Weinberger and Lumpkin, 2005; Fan et al., 2013). However, tomato crops can be infected by disease-causing bacterial, fungal, and viral pathogens that reduce yields, fruit quality, shelf-life, and nutritional content. In extreme cases, these diseases force farmers to abandon tomato production altogether. In the absence of resistant cultivars, farmers often depend on pesticides to control diseases. High reliance on pesticides poses health hazards to farmers and their families, the environment, and consumers; intensive pesticide use also can substantially increase production costs, which increase farmer financial risks and pass the accrued higher costs to consumers (Wilson and Tisdell, 2001). Resistant cultivars are among the cheapest, simplest, and most environmentally safe ways to manage disease.

Many diseases affect tomato in the tropics and subtropics, but three of the most important in terms of widespread incidence and potential to cause high yield losses include tomato yellow leaf curl disease caused by whitefly-vectored begomoviruses (Hanssen et al., 2010; Navas-Castillo et al., 2011), bacterial wilt caused by *Ralstonia solanacearum* (Hayward 1991; Mansfield et al., 2012), and late blight caused by *Phytophthora infestans* (Mont.) De Bary (Fry, 2008; Nowicki et al., 2012). The pathogens causing these diseases are genetically diverse with vast potential to generate new forms (Hayward, 1991; Fry, 2008). Most disease resistance in commercial tomato cultivars is conditioned by single genes, each conferring resistance to a specific pathogen or pathogen race, strain, or phylotype (Yang and Francis, 2007; Scott and Gardner, 2007; Scott, 2007). Six tomato yellow leaf curl disease resistance genes (*Ty-1/Ty-3*, *Ty-2*, *Ty-4*, *Ty-5*, *Ty-6*) are available in cultivated tomato (Ji et al., 2007a,b,c; Verlaan et al., 2013; Hutton and Scott, 2015). Five late blight resistance genes were introgressed from *S. pimpinellifolium* into cultivated tomato (Nowicki et al., 2012) and *Ph-2* and *Ph-3* have been used in commercial cultivars (Zhang et al., 2014). Two major

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http://dx.doi.org/10.1016/j.scienta.2016.02.020
0304-4238/

bacterial wilt resistance quantitative trait loci (QTLs), *Bwr-12* and *Bwr-6*, were identified in tomato cultivar 'Hawaii 7996' (H7996) (Thoquet et al., 1996; Carmeille et al., 2006; Wang et al., 2013) and *Bwr-12* is important for resistance to Phylotype 1 (Asia) bacterial wilt strains (Wang et al., 2013). Three race-specific genes (*I*, *I–2*, *I–3*) condition resistance to the fusarium wilt pathogen (*Fusarium oxysporum* f. sp. *lycopersici*) (Scott and Gardner, 2007). The incompletely dominant gene *Sm* offers resistance to four species of the gray leaf spot pathogen (*Stemphyllium* spp.) (Scott and Gardner, 2007). Several genes condition resistance to *Tobacco mosaic virus* (TMV) in tomato and the $Tm2^2$ allele conditions resistance to multiple strains (Scott, 2007).

Effective selection for disease resistance in segregating populations requires accurate, cost-effective screening methods that permit rapid testing of thousands of plants. Common disease screening techniques include field testing under natural disease pressure, and greenhouse/growth room screening procedures in which plants are inoculated with specific pathogen strains. Field screening is appropriate when the breeding is conducted in the region where the cultivars will be released and high disease pressure can be expected. Greenhouse seedling inoculation can assess disease reactions quickly, reduce some sources of environmental variation by use of characterized pathogen strains and defined inoculum concentrations, and avoid confounding effects from other pests or diseases. Many disease resistance genes have been mapped in tomato, and molecular markers linked to these genes are available for marker-assisted selection (MAS). The choice of screening method depends upon effectiveness, availability, cost, and convenience. Disease resistance alone is insufficient to ensure farmer adoption; commercial cultivars also must possess high yield potential, early maturity, and other horticultural traits, as well as fruit quality and nutrient content. Consequently, disease resistance breeding must be conducted with selection for important horticultural and fruit characters.

Breeding inbred lines with resistance to multiple diseases is a worthy but often difficult goal. Selecting screening protocols and the sequence of trait screening, and managing segregating populations to achieve the desired outcome, can be challenging. This paper describes a three-year selection process and the sequence of field, lab, greenhouse and molecular marker protocols applied by AVRDC-The World Vegetable Center (AVRDC) to a segregating population, which led to the development of fresh market tomato lines resistant to late blight, tomato yellow leaf curl disease, bacterial wilt, fusarium wilt, gray leaf spot, and *Tobacco mosaic virus* (TMV).

2. Materials and methods

2.1. Parents and cross

A three-parent cross, [(CLN2777G × G2-6-20-15B) × LBR-11], coded CLN3241 was created at AVRDC in 2006–2007 to develop tropically adapted, multiple disease resistant lines. CLN2777G is homozygous for resistance genes *Bwr-12* (bacterial wilt), *Ty-2* (tomato yellow leaf curl disease), and $Tm2^2$ (TMV). G2-6-20-15B is homozygous for *Ty-3* (tomato yellow leaf curl disease resistance). LBR-11 is an AVRDC F_6 selection from North Carolina State University F_2 population NC3220x-20 and is homozygous for resistance genes *Ph-2* and *Ph-3*, *I2* (resistance to race 2 of the fusarium wilt pathogen), and *Sm* (resistance to the gray leaf spot pathogen).

2.2. Line development

Segregating populations were managed by pedigree selection appropriate for self-pollinating crops (Fehr, 1987). Sixteen protocols to assess disease resistance, horticultural and fruit traits (Tables 1 and 2) were used to screen populations and lines. Selection and generation advance began in 2008 with a segregating triple-cross F_1 population (Table 3) and continued until completion of F_7 lines in June 2012. Selection was based on individual plant performance in the F_1 and F_2 generations and single plot progeny rows (30 plants per plot) with individual plant selection practiced within rows from the F_3 to F_7 generations. Two tomato crops were produced in southern Taiwan during the dry season (October–February and March–June) allowing two cycles of generation advance per year. Seed was sown in trays and seedlings were maintained in a plastic house for about 30 days before field transplanting. Before transplanting, seedlings were screened for resistance to one or more diseases, either by MAS or by greenhouse inoculation. Resistant plants were transplanted to the field for evaluation of fruit and horticultural traits. Intensive selection for tomato yellow leaf curl disease and late blight resistance was practiced during the F_1–F_4 generations. Tomato yellow leaf curl disease in southern Taiwan is caused by *Tomato yellow leaf curl Thailand virus* (TYLCTHV) and *Tomato leaf curl Taiwan virus* (ToLCTWV) (Tsai et al., 2011) with the highest pressure occurring from March to June and coinciding with high whitefly populations. Plants with tomato yellow leaf curl disease resistance genes identified by MAS were assessed in the field for tomato yellow leaf curl disease severity. Late blight screening relied on seedling inoculation with selected pathogen isolates, and also by MAS after gene markers for *Ph-2* and *Ph-3* became available in 2009. Greenhouse seedlings were screened for bacterial wilt resistance using drench inoculation, and by MAS after markers for *Bwr-12* became available in 2011. Fusarium wilt, TMV, and *Stemplyllium* screenings were performed with seedlings of $F_{7:8}$ lines. Selection for plant vigor, vine cover (extent to which foliage cover protects the fruit), early maturity, visual fruit load, fruit size and shape, fruit size uniformity within the fruit cluster, fruit firmness, fruit color development, and absence of fruit defects such as cracking was carried out during generation advance.

2.3. Disease resistance evaluations and confirmation

2.3.1. Late blight

Details of the protocols for inoculum preparation and inoculation are given in Chen et al. (2008). Briefly, 35-day-old seedlings were spray-inoculated with zoospore/sporangia suspensions of 5×10^4/mL of selected pathogen isolates. Inoculated seedlings were incubated in a growth room at 100% relative humidity and 20 ± 2 °C without light for the first 24 h. Afterwards, growth room conditions were maintained at 60–95% RH, a daily 14-h light period (70 μE m^2 s^{-1}) and 20 ± 2 °C. Each plant was visually scored 10 days after inoculation according to the following scale, where 0 = no symptoms; 1 = 1–5% leaf area affected, small lesions <2 mm and no stem lesions; 2 = 6–15% leaf area affected, necrosis-restricted leaf lesions and no stem lesions; 3 = 16–30% leaf area affected, coalescing leaf lesions or tiny water-soaked stem lesions; 4 = 31–60% leaf area affected, edge-expanding leaf lesions or a few small stem lesions (< 5 mm); 5 = 61-90% leaf area affected, drying leaf lesions or edge-expanding stem lesions; 6 = 91–100% leaf area affected, leaves blighting, extensive stem damage, or death. Resistant checks included WV700 (homozygous for *Ph-2*) and CLN2037B (homozygous for *Ph-3*). Entries and checks were tested for reactions to isolates Pi39Aand Pi237 in separate trials. WV700 is susceptible and resistant, respectively, to Pi39A and Pi237; conversely, CLN2037B is resistant to Pi39A and susceptible to Pi237. Plots included 12 plants and entries were arranged in a randomized complete block design (RCBD) with three replications.

2.3.2. Bacterial wilt

A detailed description of the bacterial wilt drench method is given in Hai et al. (2008). Inoculations were conducted with virulent

Table 1
Greenhouse, field, molecular marker, and laboratory protocols for selection of tomato disease resistances, horticultural traits, and fruit quality and nutrient contents, AVRDC Taiwan.

Trait	Protocol	Protocol no.	Reference
Horticultural	Visual assessment of vine cover, plant habit, fruit set, fruit shape, earliness	1	Hanson et al. (2012)
Tobacco mosaic virus resistance	Selection for green seedling hypocotyl conditioned by the *ah* gene (anthocyaninless of Hoffman) linked to *Tm2*^{2}	2	Robinson et al. (1970)
	NCTm-019CAPS marker for *Tm2*^{2}	3	See Table 2
Tomato yellow leaf curl disease resistance	P1-16 SCAR marker for *Ty-2*	4	See Table 2
	P6-25 SCAR marker for *Ty-3*	5	
	Seedling exposure to viruliferous whiteflies	6	Hanson et al. (2012)
Late blight resistance	TG328 marker for *Ph-3*	7	See Table 2
	dTG422/HinfI CAPS marker for *Ph-2*	8	
	P. infestans seedling screening Isolate Pi237 (T1,3) to select for *Ph-2*	9	Chen et al. (2008)
	Isolate Pi39A (T1,2) to select for *Ph-3*	10	
Bacterial wilt resistance	Seedling drench inoculation	11	Wang et al. (2013)
	SLM12-2,SLM12-10 markers for *Bwr-12*	12	See Table 2
Fusarium wilt resistance	Molecular marker for *I2* gene	13	See Table 2
	Fusarium wilt seedling drench inoculation	14	Sheu and Wang (2006)
Gray leaf spot resistance	CT55/DdeI CAPS marker for *Sm*	15	See Table 2
Fruit qualities and nutrients	Color, soluble solids, vitamin C, beta-carotene, lycopene	16	Hanson et al. (2004)

SCAR = sequence characterized regions; CAPS = cleaved amplified polymorphic sequence.

Table 2
Targeted resistance genes, linked molecular markers, and marker sequences used in marker-assisted selection.

Gene	Marker name	Marker type	Enzyme	Forward primer sequence 5′–3′	Reverse primer sequence 5′–3′	Annealing temperature (°C)	Reference
Ph-2	dTG422	CAPS		[a]			Mutschler lab
Ph-3	TG328	CAPS	BstN1	GGT GAT CTG CTT ATA GAC TTG GG	AAG GTC TAA AGA AGG CTG GTG C	55	Robbins et al. (2010)
Bwr-12	SLM12-2	SCAR		ATCTCATTCAACGCACACCA	AACGGTGGAAACTATTGAAAGG	55	Ho et al. (2013)
	SLM12-10	SCAR		ACCGCCCTAGCCATAAAGAC	TGCGTCGAAAATAGTTGCAT	55	
Ty-2	P1-16	SCAR		CACACATATCCTCTATCCTATTAGCTG	CGGAGCTGAATTGTATAAACACG	55	Yang et al. (2014)
Ty-3	P6-25	SCAR		GGT AGT GGA AAT GAT GCT GCT C	GCT CTG CCT ATT GTC CCA TAT ATA ACC	50	Ji et al. (2007a)
I2	I2OH	SCAR		TGGAGAGTTCCCTACACTTGAG	TTCTCTTCAAGGTAGTTGGCAG	55	Popoola et al. (2014)
Sm	CT55	CAPS	DdeI	CATCTGGTGAGGCGGTGAAGTA	TCCGCCCAAACAAAACAGTAATA	55	Ji and Scott (2009)
Tm2^2	NCTm-019	CAPS	HaeIII	AATTTGGGCATACTGACATC	GTTGCACACATTGGTTGTAG	55	Panthee et al. (2013)

[a] Primer sequences for *Ph-2* are available upon request from Dr. Martha Mutschler, College of Agriculture and Life Sciences, Cornell University, USA.

R. solanacearum strain Pss4 from Taiwan. Pss4 is classified as Phylotype 1 (Asia), race 1, and biovar 3 (Hai et al., 2008). For inoculum preparation, stored cultures were streaked on tetrazolium chloride medium. Several fluidal colonies were transferred to plates containing 523 media for multiplication at 30 °C for 24 h. Bacterial cells were harvested, suspended in water, and adjusted to OD600 = 0.3, (about 10^8 CFU/mL). Four-week-old seedlings grown in 2-in. pots, approximately at the five-leaf stage, were inoculated without wounding the roots by pouring 20 mL of 1×10^8 inoculum on the soil surface at the base of each plant. Checks included L390 (susceptible) and H7996 (homozygous for *Bwr-12* and *Bwr-6*). Plots included 20 plants and were arranged in a RCBD with three replications. Plants in each plot were evaluated for wilting at weekly intervals for four weeks beginning one week after inoculation. Wilted plants died, so plants were scored as healthy or wilted and the percentage of wilted plants per plot was determined after the last evaluation.

2.3.3. Tomato yellow leaf curl disease

CLN3241-coded $F_{7:8}$ lines and inbred line checks 'Tanya' (susceptible), CLN2498D (*Ty-2*) and CLN3552B (*Ty-3* and *Ty-2*) were screened in separate nethouse trials for resistance to ToLCTWV or *Tomato yellow leaf curl Thailand virus–Taiwan strain* (TYLCTHV-[TW]). Plots of entries and checks comprised 12 plants and were arranged in a RCBD with two replications. Entries in the TYLCTHV-[TW] and ToLCTWV trials were sown on 11 March and 24 March 2015, respectively. On 30 March, seedlings in the TYLCTHV trial were transferred to a nethouse containing plants of susceptible line CL5915-93D4-1-0-3 infected with TYLCTHV-[TW] and abundant viruliferous whiteflies. Similarly, on 10 April the seedlings in the ToLCTWV trial were transferred to a nethouse containing CL5915-93D4-1-0-3 plants infected with ToLCTWV and abundant viruliferous whiteflies. Every seven days after whitefly exposure, entries in both trials were scored for disease severity using a 1–6 severity scale, where: 1 = healthy, no observable symptoms; 2 = very mild with slight yellowing and mosaic on top leaves and no leaf curling; 3 = mild yellowing, mosaic and/or slight leaf curling on youngest leaves, severe symptoms; 4 = moderate yellowing and/or leaf curling on the youngest (top) leaves; 5 = severe yellowing and blistering and/or severe leaf curling plus some leaf size reduction on the youngest leaves of the main stem and/or at least one branch; 6 = very severe yellowing, blistering and/or very severe leaf curling, leaf deformation, leaf size reduction and stunting.

2.3.4. Fusarium wilt

The CLN3241-coded $F_{7:8}$ lines and check differentials were assessed in separate trials for resistance to race 1 and race 2 of *F. oxysporum* f. sp. *lycopersici* according to Sheu and Wang (2006). Checks included Bonny Best (susceptible), UC 82-L (race 1 resistance conferred by *I* gene) and Florida MH-1 (*I* gene and race 2 resistance conferred by *I-2* gene). AVRDC isolates Fol-11A (race 1) and Fol-34-1 (race 2) were used for inoculation. The inoculum was

Table 3
Targeted traits, screening sequence, and screening methods applied during generation advance in development of multiple disease resistant tomato lines, AVRDC Taiwan, 2008–2013.

Generation	No. plants/population	Period	Targeted traits	[a]Protocol no.
F_1	60 plants	October, 2008–February, 2009	*Tobacco mosaic virus* (TMV) resistance gene *Tm2*^{2} by morphological marker	2
			Horticultural (vine cover, plant habit, fruit set, fruit shape, earliness)	1
			Tomato yellow leaf curl disease (TYLCD) resistance genes *Ty-2*, *Ty-3* by marker-assisted selection (MAS) and seedling exposure to viruliferous whiteflies	4,5,6
F_2	4 populations (200 plants/population)	March–June, 2009	Horticultural	1
			TYLCD resistance genes *Ty-2*, *Ty-3* by MAS and plant exposure to viruliferous whiteflies in greenhouse and field	4,5,6
F_3	32 lines	October, 2009–February, 2010	Late blight resistance gene *Ph-3* by MAS and seedling inoculation	7,10
			Horticultural	1
F_4	28 lines	July, 2010–February, 2011	Late blight resistance gene *Ph-2* by MAS and seedling inoculation	8,9
			Horticultural	1
F_5	36 lines	March–June, 2011	Late blight resistance genes *Ph-2*, *Ph-3* by MAS	7,8
			Horticultural	1
			TYLCD resistance by plant exposure to viruliferous whiteflies in greenhouse and field	6
F_6	59 lines	June–July, 2011	Bacterial wilt resistance by drench inoculation screening	11
F_6	16 lines	October, 2011–February, 2012	Horticultural	1
			Bacterial wilt gene *Bwr-12* by MAS	12
F_7	14 lines	March–June, 2012	Horticultural	1
			Late blight resistance genes *Ph-2*, *Ph-3* by seedling inoculation	9,10
			TYLCD resistance by plant exposure to viruliferous whiteflies in greenhouse and field	6
$F_{7:8}$	5 lines	October, 2012–March, 2014	Late blight resistance genes *Ph-2, Ph-3* seedling inoculation	9,10
			Gray leaf spot resistance gene *Sm* by MAS	15
			Fusarium wilt resistance gene *I2* by MAS and seedling inoculation	13,14
			TMV (*Tm2*^{2}) by MAS	3
			Horticultural	1
			Fruit quality, nutrients	16

[a] Protocols given in Tables 1 and 2.

prepared by blending 7-day-old potato dextrose agar plates with distilled water (125 mL per plate). Two- week-old tomato seedlings were uprooted and root-dipped in the fungal culture slurry for 5 min and then transplanted to trays containing fresh potting mixture. The inoculated plants were kept in the greenhouse with the mean temperature above 25 °C. Plots of entries and checks included 12 plants and plots were arranged in a RCBD with three replications. Each plant was visually scored three weeks after inoculation according to the following scale, where 0 = healthy; 1 = slight vascular discoloration; 2 = severe vascular discoloration usually with stunting; 3 = plant wilted beyond recovery or dead.

2.3.5. Gray leaf spot and TMV

Entries were tested by molecular markers (Table 2).

2.3.6. Genotyping

DNA was isolated from fresh young leaves using the method described by Fulton et al. (1995). Primers and assay conditions for the assessment of the genotypes at resistance gene loci are provided in the references listed in Table 2.

2.4. Evaluation of yield and horticultural traits

Two field trials (FT) were conducted at AVRDC Taiwan, the first from October 2012 to March 2013 (FT1), and the second from February–June 2014 (FT2). Entries included five CLN3241 $F_{7:8}$ lines and checks CLN2498D, 'Tanya,' and CLN3078C. Entries were replicated twice and plots were arranged in a randomized complete block design. FT1 was sown and transplanted 24 September, 2012 and 28 October, respectively. FT2 was sown on 8 February and transplanted on 18 March, 2014. Plots included a 1.5 m-wide bed with two 4.0-m-long rows per bed (24 plants). Beds were covered by gray plastic mulch and rice straw and plants were staked and pruned. A basal application of 120N–52P–100K–32 Mg kg/ha and an additional 90N–38P–74K–24 Mg kg/ha was applied over four side dressings. Pesticides were used to control insects and furrow irrigation was applied as needed. FT1 plots were harvested on 5 February, 19 February, 3 March and 18 March, 2013 and FT2 plots were harvested 2 June, 5 June and 16 June, 2014.

2.5. Fruit quality and nutrient analyses

Detailed descriptions of fruit quality and nutrient analysis protocols are given in Hanson et al. (2004). About 20 fruit sampled

from the second harvest of each plot of each trial were provided to the AVRDC Nutrition lab for analysis of soluble solids, color, ascorbic acid, lycopene, and beta-carotene. Color was measured by a colorimeter on three scales represented as a, b and L. Color values of fresh tomato slurry were calculated as a/b. Soluble solids concentration was measured with a digital refractometer and presented as °brix. Carotenoid analyses were performed using high-performance liquid chromatography (HPLC, Waters, Mass.) equipped with a 717 plus autosampler, 600 controller, 2487 detector (read at 436 nm) with a 125 × 4 mm LiChrospher® 100 RP-18e column, 5 μm (Merck, Darmstadt, Germany) under isocratic conditions at ambient temperatures. The mobile phase was acetoniltrile: methanol (75:25, v/v) at a flow rate of 1.5 mL min^{-1}. Commercial β-carotene and lycopene were used as standards. The determination of total ascorbic acid was on the basis of coupling 2,4-dinitrophenylhydrazine (DNPH) with the ketonic groups of dehydroascorbic acid through the oxidation of ascorbic acid by 2,6-dichlorophenolindophenol (DCPIP) to form a yellow–orange color in acidic conditions. Commercial L-(+)-ascorbic acid was used for calibration.

2.6. Statistical analyses

Data (plot means) of disease evaluation experiments were subjected to analysis of variance appropriate for a RCBD using SAS Online Version 9.4 software (SAS Institute, Inc., Cary, N.C.). Bacterial wilt data (percent healthy plants) were transformed by arc sine square root before analysis. Yield and yield components, horticultural traits, fruit qualities and nutrients were subjected to analysis of variance (ANOVA) for each trial and over trials with the SAS General Linear Model (GLM) procedure. For the combined analysis, a mixed effects model was applied where trials, replications, and trial-by-entry were considered random effects and entry as a fixed effect.

3. Results

3.1. Line development

Screening, selection, and advance from F_1 to F_7 generations were conducted at AVRDC Taiwan over three years using a combination of field evaluation, MAS, and seedling disease screening (Tables 1–4) to select for disease resistance, yield, horticultural traits, and fruit qualities. The triple cross segregated for six disease resistances (late blight, tomato yellow leaf curl disease, fusarium wilt, gray leaf spot, TMV, bacterial wilt) and two resistance genes each for late blight (*Ph-2*, *Ph-3*) and tomato yellow leaf curl disease (*Ty-2*, *Ty-3*). Generation advance began with controlled screening of seedlings inoculated in the greenhouse/growth room, or with MAS. The three-week pre-transplant period was sufficient to screen and eliminate susceptible plants, and identify resistant plants for field transplanting. The order and frequency of screening depended on trait importance, structure of the triple cross, ease and reliability of the screening method, and availability of molecular markers.

3.2. Disease resistance evaluations

3.2.1. Late blight

Checks CLN2037B (*Ph-3*), WV700 (*Ph-2*), and susceptible parents G2-6-20-15B and CLN2777G showed expected reactions to isolates *Pi*-237 and *Pi*39A after seedling inoculation. The five CLN3241-prefixed lines and LBR-11 were resistant (scores ≤2.0) to both isolates (Table 4) and subsequent marker analysis confirmed that these entries were homozygous for *Ph-2* and *Ph-3*.

3.2.2. Bacterial wilt

Check lines H7996 (resistant) and L390 (susceptible) showed expected reactions after drench inoculation (Table 4). The five F_7 test lines (CLN3241 prefixes) and parent CLN2777G tested positive by marker analysis for *Bwr*-12; % wilted plant means of the five resistant lines ranged from 15 to 35 and all were significantly greater than susceptible parents G2-6-20-15B and LBR-11 with wilted plant means ≥97%.

3.2.3. Tomato yellow leaf curl disease

Susceptible check 'Tanya' developed severe symptoms (severity score >5.0) after infection with TYLCTHV-[TW] or ToLCTWV (Table 4) while CLN2498D developed mild (severity score <3.0) and moderate (severity score ~4.0) symptoms after ToLCTWV and TYLCTHV-[TW] infection, respectively. The five CLN3241 lines (homozygous for both *Ty-3* and *Ty-2* or only *Ty-3*) developed mild (severity score <3.0) symptoms to both begomoviruses.

3.2.4. Fusarium wilt

The CLN3241-prefixed lines and parents were highly resistant to race 1 based on seedling inoculation (Table 4). The gene *Immunity* (*I*) is common in commercial tomato cultivars and probably all entries were homozygous for *I*, but AVRDC lacks a marker for *I* and its presence could not be confirmed. The five test lines and parents G2-6-20-15B and LBR-11 were resistant to race 2 and also tested positive for the *I-2* gene by marker I2OH.

3.2.5. Gray leaf spot

Analysis with marker CT55 revealed presence of the *Sm* resistance gene in parents CLN2777G and LBR-11, and in all CLN3241-coded lines.

3.2.6. TMV

The NCTm-019 marker indicated that parent CLN2777G and the five CLN3241-coded lines were homozygous for $Tm2^2$. These lines have green hypocotyls conditioned by the recessive allele 'anthocyaninless of Hoffman' (*ah*), which is in coupling phase linkage with $Tm2^2$ (=$Tm2^a$) (Robinson et al., 1970).

3.3. Line evaluation: yield, horticultural traits, fruit quality and nutrient content

The combined analysis over trials showed significant entry-trial interactions for most variables and entry means for FT1 and FT2 are presented (Table 5). Analysis of variance revealed significant entry mean squares in both trials for yield, average fruit weight, solids, pH, and vitamin C (data not shown). Entry mean squares for average fruit number per plant, days to maturity, solids, color and beta-carotene were significant or highly significant in FT1 or FT2, but not in both. Mean squares for lycopene and acid were non-significant for both trials. Favorable conditions for tomato production during FT1 (mean daily 26.9 °C/15.4 °C maximum/minimum temperatures; total rainfall of 125 mm) resulted in an average fruit yield of 104 t/ha (Table 5). In contrast, higher temperatures (29.2 °C/21.5 °C maximum/minimum temperatures), higher total rainfall (343 mm) and tomato yellow leaf curl disease pressure during FT2 led to an average mean entry yield of 28 t/ha. Average fruit weights were consistent across trials but a 63% reduction in average fruit number per plant contributed to lower yields in FT2. Number of days to maturity (number of days from transplanting to harvest) was 38 days shorter in FT2 than FT1. Late blight infection and bacterial wilt were absent in both trials but high tomato yellow leaf curl disease symptoms developed on susceptible 'Tanya' and CLN2498D during FT2. Values for solids fell within the normal range for fresh market tomato and were consistent between trials. Mean fruit color (a/b = 1.97) in FT1 indicated

Table 4
Disease resistances of five F_7-derived F_8 lines, parents, and checks determined by seedling inoculations and/or molecular markers.

Entry	Entry type	Tomato yellow leaf curl disease (TYLCD)[1]				Late blight (LB)[2]				Fusarium wilt (FW)[3]			Bacterial wilt (BW)[4]	
		Ty-3 P6-25	*Ty-2* P1-16	ToLCTWV Severity score	TYLCTHV	*Ph-2* dTG422	Pi-237 Score	*Ph-3* TG328	Pi-39A Score	Fol-11A (Race 1) DSR	Fol-34-1 (Race 2)	*I2* I2OH	*Bwr-12* SLM12-2 SLM12-10	BW%
CLN3241S	$F_{7:8}$ selection	+	–	2.5 b	2.0 c	+	0.9 d	+	1.8 de	0.0 c	0.3 cd	+	+	35 a–c
CLN3241R	$F_{7:8}$ selection	+	–	2.1 b–d	2.0 c	+	0.8 d	+	1.2 e	0.0 c	0.2 c–e	+	+	47 c
CLN3241P	$F_{7:8}$ selection	+	+	1.2 e	2.0 c	+	0.9 d	+	1.3 e	0.0 c	1.9 b	–	+	35 a–c
CLN3241Q	$F_{7:8}$ selection	+	+	1.5 de	1.8 c	+	1.2 cd	+	1.7 de	0.0 c	0.0 e	+	+	7 ab
CLN3241H-27	$F_{7:8}$ selection	+	+	1.5 de	2.0 c	+	0.8 d	+	1.4 de	0.0 c	0.03 de	+	+	15 a–c
LBR-11	Parental line	–	–	–	–	+	1.5 c	+	2.2 dc	0.0 c	0.1 c–e	+	–	97 d
G2-6-20-15B	Parental line	+	–	–	–	–	3.9 b	–	3.5 ab	0.0 c	0.03 de	+	–	100 d
CLN2777G	Parental line	–	+	–	–	–	5.2 a	–	3.8 a	0.0 c	1.9 b	–	+	40 bc
Tanya	TYLCD check (susceptible)	–	9	5.5 a	5.3 a									
CLN2498D	TYLCD check (*Ty-2* gene)	–	+	2.2 bc	3.9 b									
CLN3552B	TYLCD check (*Ty-3* and Ty-2 genes	+	+	2.1 b–d	2.1 c									
H7996	BW check (*Bwr-12* gene)												+	12:00 AM
L390	BW check (susceptible)												–	97 d
WV700	LB check (*Ph-2* gene)					+	1.0 cd	–	2.7 bc					
CLN2037B	LB check (*Ph-3* gene)					–	4.9 a	+	1.5 de	2.1 b	0.1 c–e			
Bonny Best	FW check (susceptible)									3.0 a	2.9 a			
UC82-L	FW check (*I* gene)									0.1 c	2.0 b			
MH-1	FW check (*I2* gene)									0.0 c	0.0 e		–	–
Significance of the Entry mean square				**	**		**		**	**	**			**

[1] *Ty-3* and *Ty-2* genes condition TYLCD resistance and presence (+) or absence (–) was determined by molecular markers P6-25 (*Ty-3*) and P1-16 (*Ty-2*). Marker primer sequences are given in Table 2. ToLCTWV (*Tomato leaf curl Taiwan virus*) and TYLCTHV (*Tomato yellow leaf curl Thailand virus*) are begomoviruses that cause TYLCD. Severity scores were determined six weeks after seedling exposure to viruliferous whiteflies and ranged from 1 (healthy, no symptoms) to 6 (severe stunting, leaf curling, yellowing).

[2] *Ph-2* and *Ph-3* confer resistant and susceptible reactions, respectively, to pathogen isolate Pi-237; conversely, *Ph-2* and *Ph-3* produce susceptible and resistant reactions, respectively, to isolate Pi39A. Presence (+) or absence (–) of *Ph-2* and *Ph-3* was determined by markers dTG422 and TG328, respectively. Seedlings were inoculated in an AVRDC growth room and disease scores ranged from 0 (no symptoms) to 6 (91–100% of leaf area affected, extensive stem damage, and/or plant dead).

[3] *I2* resistance gene confers resistance to race 2 of the FW pathogen and its presence (+) or absence (–) was determined by marker I2OH. Seedlings were screened for FW races 1 and 2 by root-dip inoculation. DSR = disease severity rating where values ranged from 0 (healthy) to 3 (plant wilted beyond recovery or dead).

[4] *Bwr-12* gene conditions resistance to the BW pathogen and its presence (+) or absence (–) was determined by markers SLM12-2 and SLM12-10. Marker primer sequences are given in Table 2. BW%: is percent wilted plants after drench inoculation with *Ralstonia solanacearum* isolate Pss4. Data were transformed by arc sine square root prior to analysis of variance and mean separation. Non-transformed means are shown.

** Significant at $P < 0.001$.

Table 5
Evaluation of multiple disease resistant tomato lines for yield, horticultural, and fruit traits in early and late dry season trials, AVRDC Taiwan.

Entry code	Fruit weight (g)		Fruit no. per plant		Maturity days after transplanting		Yield (t/ha)		Solids (°Brix)		Color[1] *a*/*b*		Vitamin C mg/100 g fresh weight		Beta-carotene		Lycopene	
	FT1	FT2	FT1	FT2	FT1	FT2	FT1	FT2	FT1	FT2	FT1	FT2	FT1	FT2	FT1	FT2	FT1	FT2
CLN3241S	131 a	136 a	28	8 d	116 bc	78	116 a	25 bc	4.2	4.7 a	1.78 c	1.27	11.7 bc	19.2 b	0.26	0.33 b	5.93	3.62
CLN3241R	133 a	133 a	31	9 cd	116 bc	78	110 a	25 bc	4.3	4.8 a	1.77 c	1.51	10.4 c	19.6 b	0.31	0.34 b	5.53	4.99
CLN3241P	76 b	84 b	39	19 ab	117 ab	78	111 a	34 a–c	4	3.8 b	1.89 bc	1.15	19.4 ab	20.2 b	0.29	0.28 bc	5.6	3.12
CLN3241Q	75 b	86 b	46	19 ab	117 ab	78	107 a	42 a	3.8	3.6 b	1.88 bc	1.01	16.3 bc	18.7 b	0.26	0.45 a	5.07	2.77
CLN3241H-27	76 b	90 b	43	20 a	116 bc	78	121 a	40 a	3.8	3.8 b	1.84 c	0.97	16.0 bc	20.6 b	0.23	0.30 bc	5.53	3.23
Tanya	69 b	59 c	40	11 cd	118 a	79	65 b	11 e	3.8	4.2 ab	2.05 ab	1.69	12.6 bc	18.5 b	0.48	0.22 c	5.6	6.04
CLN2498D	70 b	49 c	41	12 b–d	115 c	80	101 a	16 de	3.7	3.5 b	2.14 a	0.97	16.8 bc	23.3 b	0.41	0.29 bc	7.34	2.58
CLN3078C	82 b	88 b	35	16 a–c	118 a	79	101 a	34 ab	4.2	4.8 a	2.08 a	1.64	27.4 a	37.7 a	0.4	0.34 b	5.06	5.69
Mean	89	90	38	14	116	78	104	28	3.95	4.1	1.93	1.27	16.3	22.2	0.33	0.32	5.71	4
Entry mean square	**	**	ns	*	*	ns	*	**	ns	*	**	ns	*	**	ns	*	ns	ns

FT = field trial. FT1 (dry season) and FT2 (early rainy season) were conducted October 2012–March 2013 and February–June 2014, respectively.
Means within columns followed by the same letter are not significantly different by least significant difference ($P < 0.05$).
[1] Values for *a* and *b* were measured with a chromometer using a red standard surface. Immature green tomatoes have *a*/*b* ratio less than 0. The *a*/*b* ratio increases to zero and above as the fruits ripen toward a dark red. Values > 2.0 have superior color.
* Significant at $P < 0.05$.
** Significant at $P < 0.01$.

a moderately deep red internal color favored by most consumers; however, mean entry fruit color in FT2 (*a*/*b* = 1.27) fell by 34%, which was due to a 29% reduction in lycopene content between trials. Lycopene synthesis is temperature sensitive and is significantly reduced above 28 °C (Tomes, 1963). Mean beta-carotene contents (provitamin A) were consistent over trials while vitamin C content was 36% higher in FT2 versus FT1. Vitamin C is an antioxidant and higher temperatures and sunlight during FT2 may have induced greater vitamin C synthesis.

CLN3241S, CLN3241R, CLN3241P, CLN3241Q, and CLN3241-H27 have semi-determinate plant habits producing fair to good foliage cover; moderate pruning is recommended depending on plant spacing. Each line produced deep-globe shaped, moderately firm fruit with uniform (*u* gene) shoulders. Fruit pedicels were jointed. Fruit of CLN3241S and CLN3241R had 5–7 locules, while CLN3241H-27 and CLN3241P had 2–3 locules. All lines tended to develop rain check (small cracks in the cuticle) under high rainfall and relative humidity.

4. Discussion

This study describes the integrated application of molecular markers and field and greenhouse screening methods including appropriate checks to develop multiple disease resistant tomato lines. This achievement was possible through multidisciplinary collaboration between breeders, plant pathologists, and molecular breeders. The multiple disease resistant tomato lines could be valuable in mid-altitude tropical production areas such as the East African highlands, parts of south India, or Central America, where late blight, tomato yellow leaf curl disease, and bacterial wilt commonly occur sequentially or simultaneously. Yields of the five lines were high under optimal temperatures but much reduced under high temperatures and rainfall, and thus are not recommended for hot rainy season production in the lowland tropics. Seed of three of the five lines is available from AVRDC (http://avrdc.org/seed/improved-lines/); these lines have potential for release as inbred line cultivars, hybrid parental lines, or as breeding stock.

Effective and inexpensive screening methods enable efficient selection of plants carrying desired combinations of resistance genes. Different screening methods were employed during generation advance including field evaluation under natural disease pressure, seedling inoculation with selected pathogen isolates, one morphological marker, and DNA markers linked to disease resistance genes. AVRDC tomato disease resistance screening has been based mainly on seedling inoculation, but MAS has become increasingly important. Molecular markers enabled discernment of plants with both *Ty-3* and *Ty-2* genes versus plants with *Ty-3* alone—a distinction not possible based on symptoms alone after exposure to the leaf curl begomoviruses prevalent in Taiwan. Field screening under high tomato yellow leaf curl disease pressure was important to confirm that lines with *Ty* resistance genes expressed high levels of resistance. Late blight screening in an early generation of this population was performed by sequential screening with selected pathogen isolates targeting *Ph-2* and *Ph-3* resistance genes. Molecular markers for *Ph-2* and *Ph-3* were used for screening after they were made available to AVRDC by Martha Mutschler of Cornell University, and close linkage between the markers and resistance genes was confirmed. The *Ph-2* and *Ph-3* markers were advantageous because individual plants could be assayed for both *Ph-2* and *Ph-3*, and homozygotes versus heterozygotes could be distinguished. Expression of bacterial wilt resistance is affected by pathogen strain and environmental factors such as temperature, moisture, and nematode infection (Hayward, 1991). In the past, selection for bacterial wilt resistance at AVRDC was delayed until the F_3 or later generations, when the lines were screened multiple times during generation advance to confirm resistance. Availability of markers for *Bwr-12* enabled identification of lines carrying this resistance gene.

Broad and durable resistance to a wide range of pathogen races/strains is a desired but often elusive objective of resistance breeding. Durability of resistance is usually known only in hindsight; it is affected by multiple factors such as the extent and time to which a particular resistance gene has been commonly deployed in cultivars, the frequency in which production environments favor the pathogen and disease (Kang et al., 2005), and the factors affecting pathogen populations such as mutation rates, gene flow, reproduction and mating systems, spore persistence, and selection pressure (McDonald and Linde, 2002). The partially dominant gene *Sm* that confers resistance to four *Stemphyllium* species has been incorporated in many tomato cultivars grown throughout the world without reports of resistance breakdown for more than 60 years (Parlevliet, 2002). TMV resistance conferred by the single dominant gene $Tm2^2$ (or $Tm2^a$) also has remained durable (Harrison, 2002; Kang et al., 2005; Scott, 2007) despite many years of widespread use in tomato cultivars. $Tm2^2$ inhibits viral cell-to-cell movement and two mutations in the pathogen are required to break resistance (Harrison, 2002).

Durable resistance to the late blight pathogen and begomoviruses poses major challenges to breeders and pathologists. Both pathogens are highly diverse, with the capacity to create new forms through recombination and migration. Consequently, a gene pyramiding strategy to combine multiple and complementary resistance genes has been suggested to improve chances of conferring durable resistance to these diseases (Ji et al., 2007b; Vidavski et al., 2008; Nowicki et al., 2012). All CLN3241-prefixed lines are homozygous for the incompletely dominant gene *Ty-3* and three lines were homozygous for *Ty-3* and *Ty-2*. *Ty-3* exhibited high levels of resistance to *Tomato yellow leaf curl Thailand virus* (TYLCTHV) and *Tomato leaf curl Taiwan virus* (ToLCTWV), multiple bipartite begomoviruses in Guatemala (Garcia et al., 2008), and to monopartite and bipartite begomoviruses in India (Prasanna et al., 2014). AVRDC gives high priority to incorporation of *Ty-3* into its new breeding lines. Our results indicate that *Ty-3* reduced tomato yellow leaf curl disease symptom severity, but did not prevent virus infection. Blocking virus infection by pyramiding *Ty-3* with additional *Ty* genes is desirable to reduce begomovirus inoculum and possible co-infection by multiple viruses and potential recombination. The *Ty-3* + *Ty-2* combination did not eliminate virus infection in this study and pyramiding new resistance gene combinations such as *Ty-3*, *Ty-5* and *Ty-2* should be explored.

P. infestans is notorious for its capacity to quickly overcome resistant cultivars (Fry, 2008). Pathogen diversity and disease pressure will increase with migration and introduction of new clonal lineages and the A2 mating type (Fry, 2008). The CLN3241-prefixed lines are homozygous for the dominant gene *Ph*-3 and the partially dominant gene *Ph-2*. Both genes are race-specific (Chen et al., 2008), but *Ph-2* to a lesser extent than *Ph-3* (Nowicki et al., 2012). Their resistances complement each other in pathogen race protection (Chen et al., 2008). Resistance and durability of cultivars with *Ph-2* and *Ph-3* will depend on the prevalent pathogen clonal lineages in a particular region and whether both A1 and A2 mating types are present. Tomato cultivars with *Ph-3* alone have been effective in Tanzania (Ojiewo et al., 2010) and probably other East African countries where *P. infestans* isolates are derived from the US-1 clonal lineage (Pule et al., 2013). Cultivars with *Ph-2* and *Ph*-3 resistance generally have held up well in the USA (Mutschler, personal communication) but not in Taiwan after displacement of US-1 by isolates of the US-11 clonal lineage, beginning around 2004 (Chen et al., 2008). Tomato breeders continue to search for new late blight resistance genes in wild tomato species *S. pimpinellifolium* (Merk et al., 2012) and *S. habrochaites* (Brouwer and Clair, 2004; Li et al., 2011), but introgression of new alleles from wild species into commercial cultivars requires many years. Farmers should be encouraged to adopt sanitation, fungicides, and other control tactics to help prolong the durability of late blight resistance.

Linkage of resistance genes can facilitate or hinder gene pyramiding, depending on whether resistances are linked in a coupling or repulsion phase. The *I-2* gene for resistance to race 2 of the fusarium wilt pathogen was mapped to the bottom of chromosome 11 (Hanson et al., 2000; Ji et al., 2009,) and it is located about 400,000 base pairs below the *Ty-2* locus for begomovirus resistance (Yang et al., 2014). The parental lines of the triple cross used in this study carried these genes in a repulsion phase configuration with genotypes *Ty-2*/*i2*$^{+}$ (CLN2777G) or *ty-2*$^{+}$/*I2* (G2-6-20-15B, LBR-11). Crossover between the *Ty-2* and *I–2* loci occurred and resulted in one or more recombinants in a coupling phase linkage (*Ty-2*/*I2*); CLN3241H-27, CLN3241P, and CLN3241Q were homozygous for *Ty-2* and *I2*. MAS has been applied to identify coupling phase resistance gene recombinants, such as was done for *Ph-3* and *Sw-5* (Robbins et al., 2010). Identification of *Ty-2* and *I2* recombinants in this study did not result from a directed search, and the coupling phase linkage was not detected until the $F_{7:8}$ lines were screened for fusarium wilt resistance; the *Ty-2* and *I2* crossover event would have been rare (Yang et al., 2014). The three lines homozygous for *Ty-2* and *I2* were derived from the same F_2 plant, so the crossover occurred in the F_2 or F_1. This is the first report of tomato lines with *Ty-2*/*I2* in the coupling phase linkage—a significant finding, because both genes will co-segregate in future crosses.

5. Conclusions

The multiple disease resistance program described here began in 2007 when selection at AVRDC relied mainly on field and greenhouse disease screening. Molecular markers linked to the targeted resistance genes became available during the three-year line development period and confidence in the markers increased after multiple comparisons between marker and seedling screening results. Effective markers are now available for all disease resistance genes targeted in this study and for many other important genes. AVRDC and many tomato breeding programs routinely apply MAS to large segregating populations, especially the F_2, which increases breeding efficiency through identification of multiple homozygotes and early elimination of susceptible genotypes, and focuses labor-intensive and costly field evaluation on high potential lines. Nevertheless, biological screening to confirm disease resistance in advanced inbred lines will still be a critical step in the development of multiple disease resistant tomato.

Acknowledgements

The authors thank Rose Chan for formatting assistance and Maureen Mecozzi for editing the manuscript. Financial support from the Federal Ministry for Economic Cooperation and Development, Germany, project number 11.7860.7-001.00 and 03.7860.4-001.00 is gratefully acknowledged. Core funding to support AVRDC activities worldwide is provided by the Republic of China (ROC), UK Department for International Development (UK/DFID), United States Agency for International Development (USAID), Germany, Thailand, Philippines, Korea, and Japan.

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