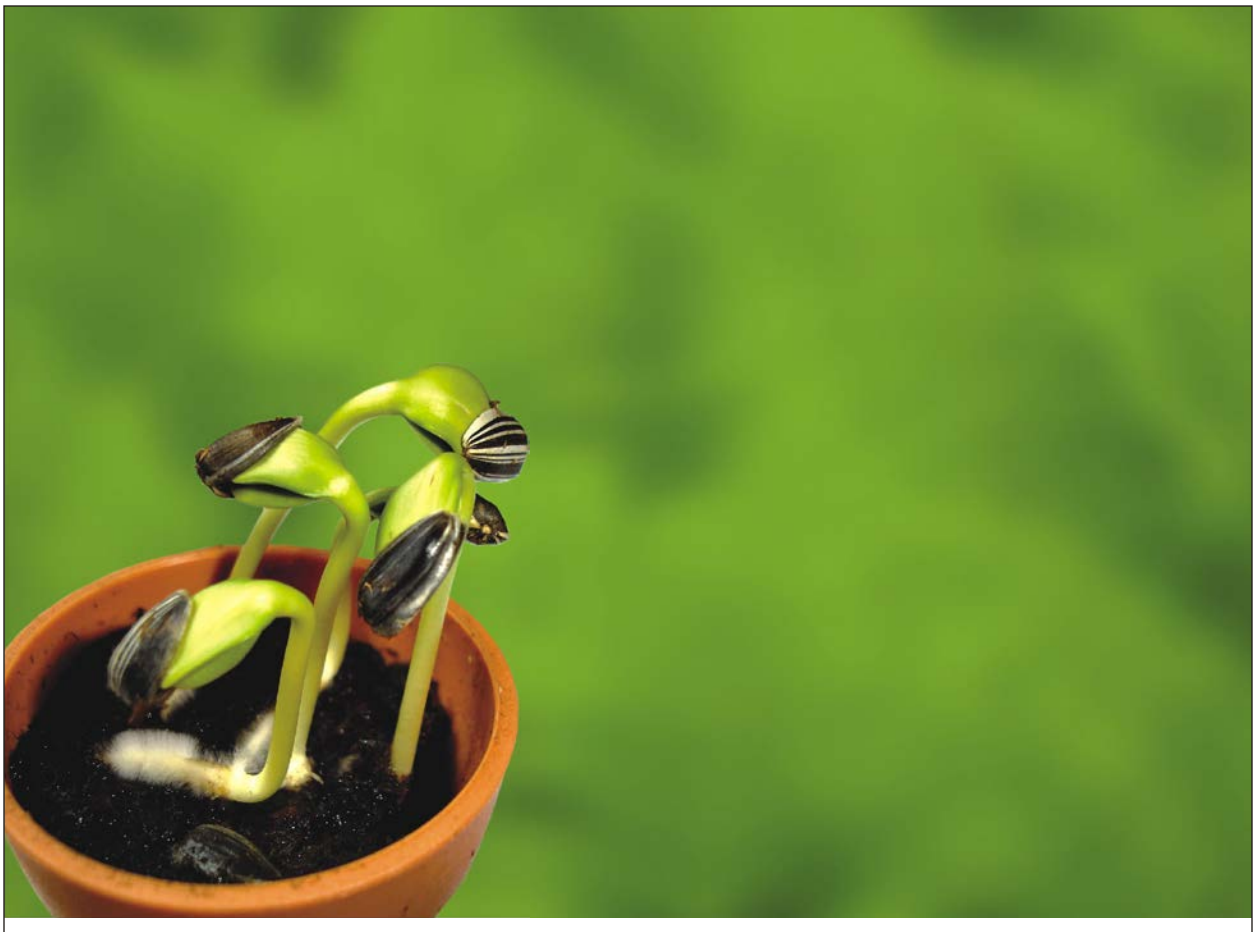

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Multiplex PCR for Detection of Tomato Yellow Leaf Curl Disease and Root-Knot Nematode Resistance Genes in Tomato (*Solanum lycopersicum* L.)

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ABSTRACT

Tomato yellow leaf curl virus and root-knot nematodes cause diseases in tomato that can lead to heavy production losses. Resistance genes against both pathogens are available and used in breeding. Molecular markers for resistance gene alleles greatly enhance selection of resistant plants in breeding. In order to make marker-assisted selection for the most commonly used resistance genes in tomato breeding more effective, we have developed a multiplex Polymerase Chain Reaction (PCR) assay to simultaneously assess the genotype at four resistance loci. For this purpose, we have selected available markers for the tomato yellow leaf curl disease resistance gene loci *Ty-2* and *Ty-5* and for the root-knot nematode resistance gene *Mi-1* and have developed a new marker for the tomato yellow leaf curl disease resistance gene locus *Ty-1/3* to be incorporated in a multiplex PCR assay. The assay correctly predicted the genotypes of tomato breeding lines known to be homo-or heterozygous for the resistance or susceptibility alleles at the *Ty-1/3*, *Ty-2*, *Ty-5* and *Mi-1* gene loci. In wild tomato (*Solanum chilense*) accessions LA1969 and LA1932, the markers failed to identify alleles conditioning susceptibility at the *Ty-2* and *Ty-5* loci. Replacement of a single marker of the multiplex assay by another marker was possible without affecting the accurateness of the assay, as long as the size differences of the DNA fragments of the markers were sufficiently large. Combining four markers for resistance alleles commonly used in tomato breeding into a multiplex assay offers a significant cost reduction for marker-assisted breeding in tomato.

Key words: Marker-assisted selection, multiplex PCR, tomato yellow leaf curl disease resistance, root know nematode resistance

INTRODUCTION

Molecular markers associated with favorable alleles have become important tools in plant breeding. Markers linked with disease resistance genes can be used to select resistant plants at early stages in the absence of the pathogen and can facilitate pyramiding of multiple resistance genes in a genotype. Marker-assisted selection allows for smaller breeding populations and accelerates the generation of plants with desirable alleles, thereby reducing the cost of breeding resistant plants (Slater *et al*., 2013).

Tomato (*Solanum lycopersicum* L.) is the most economically important horticultural crop with annual worldwide production of more than 160 million tons in 2012 (FAO., 2014) and an economic value of US$ 1.9 billion in the USA alone (USDA., 2013). Tomato served as a model crop to develop the concept of molecular marker-based selection in breeding (reviewed by Foolad and Panthee, 2012). A multitude of disease resistance and quality-related genes have been identified and mapped in wild and cultivated tomato species (Foolad and Panthee, 2012; Hajjar and Hodgkin, 2007); some of these genes, such as *Tomato yellow leaf curl virus* and root-knot nematode resistance genes, are frequently used in tomato breeding and the seed industry routinely applies marker-assisted selection for these genes.

Tomato yellow leaf curl disease is a major limiting factor for tomato production worldwide. Tomato yellow leaf curl is caused by a whitefly-transmitted Begomovirus (family Geminiviridae). Since the whitefly vector is difficult to control and tomato yellow leaf curl resistance is absent in the cultivated tomato gene pool, emphasis was given to identify host resistance against this disease in wild tomato species such as *S. chilense, S. habrochaites* and *S. peruvianum* (Zamir *et al*., 1994; Ji *et al*., 2007, 2009; Hanson *et al*., 2000, 2006; Garcia *et al*., 2007; Anbinder *et al*., 2009). Mapping of the different tomato yellow leaf curl resistance genes facilitated marker-assisted introgression of these genes into cultivated tomato. Tomato yellow leaf curl resistance genes *Ty-1* and *Ty-3*, which led to virus tolerance rather than immunity, are alleles of the same gene and encode an RNA-dependent RNA (RDR) polymerase (Verlaan *et al*., 2013). The allele conditioning susceptibility at the *Ty-1/3* locus differs from the *Ty-1* and *Ty-3* resistance alleles by a four amino acid deletion in the amino-terminal part of the RDR protein encoded by the gene Solyc06g051190. The *Ty-2* tomato yellow leaf curl resistance gene is derived from *S. habrochaites* and has been successfully used in breeding programs to provide resistance against monopartite *Tomato leaf curl Taiwan virus*, but it became ineffective after the bipartite begomovirus *Tomato yellow leaf curl Thailand virus* was introduced into Taiwan and became the predominant strain (Kenyon *et al*., 2014). The *Ty-2* locus has recently been fine-mapped to a 300 Mb region of chromosome 11, allowing sourcing of new, tightly linked markers for this locus (Yang *et al*., 2011). The recessive *Ty-5* resistance gene is derived from *S. peruvianum* accession TY172 (Anbinder *et al*., 2009) and when present in the homozygous state, provides resistance against multiple mono-and bipartite begomovirus species (Hutton *et al*., 2012).

Root-knot nematodes from the genus *Meloidogyne* cause serious tomato yield losses, especially in greenhouse production (Devran *et al*., 2010). *Meloidogyne incognita*, *M. javanica* and *M. arenaria* are the most common and economically important root-knot nematode species affecting tomato cultivation. Management of root-knot nematodes by soil fumigation is feasible, but host resistance is preferred as it is an environmentally safe and user-friendly control method (reviewed by Zasada *et al*., 2010). The dominant root-knot nematode resistance gene *Mi-1* was introgressed from *S. peruvianum* into cultivated tomato (Messeguer *et al*., 1991; Roberts and Thomason, 1986). The introgressed DNA region contains several genes and pseudogenes, among which only *Mi-1.2* was shown to confer resistance to nematodes (Milligan *et al*., 1998), aphids (Rossi *et al*., 1998) and whiteflies (Nombela *et al*., 2003). Several PCR-based markers were generated for the *Mi-1* gene (Ho *et al*., 1992; Williamson *et al*., 1994); among them, the co-dominant SCAR (sequenced characterized amplified region) marker *Mi-23* tightly linked to the *Mi-1.2* gene is highly suitable for marker-assisted selection for root-knot nematode resistance in tomato (Seah *et al*., 2007).

The PCR-based markers to locate resistance alleles of the genes mentioned above are applied by many tomato breeders to monitor the presence of resistance gene alleles in breeding lines and

to pyramid multiple resistance genes into individual lines. Stacking multiple resistance genes is thought to increase the effectiveness and durability of resistance against diseases (Vidavski *et al*., 2008), but is difficult to achieve without marker-assisted selection targeting multiple resistance loci.

Combining PCR primers targeting several different resistance genes in one multiplex reaction reduces the workload and costs for marker-assisted selection (Elnifro *et al*., 2000), but might be hindered by undesirable interactions among primers and PCR fragments. Masuelli *et al*. (2000) described the development of a PCR assay simultaneously detecting the root-knot nematode resistance gene *Mi-1* and the *Tomato spotted wilt virus* resistance gene *Sw-5* in tomato. Several other multiplex assays specific for two to three tomato resistance gene alleles have been described (Chen *et al*., 2012; Fu *et al*., 2013). Liu *et al*. (2013) disclosed a multiplex PCR assay for *Ty-1*, *Ty-2*, *Mi-1* and *Cf-5* resistance genes, but not for *Ty-3* and *Ty-5*. The current study demonstrates the feasibility of a multiplex assay simultaneously detecting four highly important resistance gene loci for breeding tropical tomato, comprising in total four resistance gene alleles for tomato yellow leaf curl and one against root-knot nematodes into one PCR reaction for marker-assisted selection. Single markers of the assay could be replaced by others, as long as the obtained fragment sizes showed marked differences. Therefore, the assay can be easily adapted to specific needs of a marker-assisted selection program.

MATERIALS AND METHODS

Plant material and DNA isolation: All tomato lines were obtained from AVRDC-The World Vegetable Center's tomato breeding team. A set of 20 tomato genotypes carrying different combinations of four tomato yellow leaf curl and one root-knot nematode resistance gene allele in the homozygous state, one line susceptible to both tomato yellow leaf curl and nematodes and two wild tomato accessions LA1969 and LA1932 (*S. chilense*), carrying the *Ty-1* and *Ty-3* resistance genes, respectively, was used to develop and test the multiplex PCR assay (Table 1). Additionally, the assay was tested on individuals of two F_2 populations that were potentially heterozygous for the target genes. The CLN3900 F_2 plants were derived from a cross between the parents CLN3552F2-1-19-17-27-28 (homozygous for *Ty-1/3*)×CLN3682F1-10-9-5-12 (homozygous for *Ty-1/3*, *Ty-2* and *Mi-1*) with *Ty-2* and *Mi-1* segregating in the progeny. CLN3906 F_2 plants were obtained from cross CLN3552F2-1-19-17-27-28 (homozygous for *Ty-1/3*) with CLN3212F1-21-31-11-27-3-11-24-4 (homozygous for *Ty-5*) resulting in F_2 plants segregating for *Ty-1/3* and *Ty-5*. Young leaves were harvested from the plants and DNA was extracted according to Fulton *et al*. (1995).

Primer design for PCR-based *Ty-1/3* and *Ty-5* markers: Based on the DNA sequences of the *Ty-1* and *Ty-3* resistance alleles and the susceptible genotype at the *Ty-1/3* locus, a SCAR marker was designed targeting the 12 bp deletion of the susceptibility allele at the *Ty-1/3* locus in gene Solyc06g051190 (Verlaan *et al*., 2013). The primer sequences of the newly designed SCAR marker distinguishing the *Ty-1/3* resistance allele from the susceptibility allele are shown in Table 2. Previously, the *Ty-5* resistance locus has been tagged with a Cleaved Amplified Polymorphic Sequences (CAPS) marker SINAC1 (Anbinder *et al*., 2009). The CAPS markers are not appropriate for multiplex assays, therefore we sought a Single Sequence Repeat (SSR) marker linked to the locus and diagnostic for the *Ty-5* resistance allele. Six SSR markers available at AVRDC-The World Vegetable Center located on chromosome four between positions 2.856-3.2 Mb according to the SL2.40 tomato full genome sequence (The Tomato Genome Consortium, 2012) were tested for polymorphism between plants carrying the *Ty-5* resistance or susceptibility alleles (Table 2). For

Table 1: Verification of *TY-1*, *Ty-2*, *Ty-3*, *Ty-1/3*, *Ty-5* and *Mi-1* genotypes in a representative set of tomato germplasm including a variety, homozygote breeding lines, individuals of F_2 families and wild tomato (*S. chilense*) accessions

			Locus and marker								
			Ty-1	*Ty-3*	*Ty-1/3*	*Ty-2*	*Ty-2*	*Ty-5*	*Mi-1*	*Ph-3*	*BW12*
ID	Genotype	Variety	(TG178)	(P6-25)	(M2)	(T0302)	(TES0344)	(TM273)	(Mi-23)	(R2M1S2)	(SLM12-2)
1	Tanya	Homozygous	S	S	S	S	S	S	S	S	S
2	*Ty-52*	breeding	R	S	R	S	S	S	S	S	S
3	CLN2498D	lines	S	S	S	R	R	S	S	S	R
4	CLN3024A		R	S	S	R	R	S	S	S	R
5	CLN3205B		R	R	R	S	S	S	S	S	R
6	CLN3212C		S	S	S	S	S	R	S	S	R
7	CLN3150A-5		S	S	S	R	R	R	S	S	R
8	CLN3126A-7		S	3a	R	R	R	S	S	S	H
9	CLN3447G		S	S	S	R	R	R	S	S	R
10	CLN3070J		S	R	R	R	R	S	S	S	R
11	CLN3241H-27		R	R	R	R	R	S	S	R	R
12	CLN3125P		R	R	R	R	R	S	S	S	R
13	CLN2819B		R	R	R	R	R	S	S	S	S
14	F8-48		S	S	S	S	S	R	S	S	S
15	F9-159		S	S	S	S	S	R	S	S	S
16	CLN3682F1-10- 3-4-27-1		n.t	n.t	R	R	R	S	R	R	R
17	FLA456		n.t	n.t	S	S	S	R	S	S	S
18	CLN3125K		S	R	R	S	n.t	S	S	R	R
19	LA1969	*S. chilense*	R	n.t	R	S	n.t	S	S	R	S
20	LA1932		n.t	R	R	S	n.t	S	S	R	S
24	T-21	Homozygous breeding line	n.t	n.t	R	-	n.t	S	n.t	S	R
A1	CLN3552F2-1-	Individuals of	n.t	n.t	R	H	n.t	S	H	n.t	n.t
A2	19-17- 27-2 x	F_2 families	n.t	n.t	R	H	n.t	S	S	n.t	n.t
A3	CLN3682F1-10-		n.t	n.t	R	R	n.t	S	H	n.t	n.t
A4	9-5-12 = CLN3900		n.t	n.t	R	H	n.t	S	R	n.t	n.t
B1	CLN3552F2-1-19-17		n.t	n.t	H	S	n.t	H	S	n.t	n.t
B2	-27-2xCLN3212F1-		n.t	n.t	H	S	n.t	S	S	n.t	n.t
B3	21-31-11-27-3-11-		n.t	n.t	S	S	n.t	H	S	n.t	n.t
B4	24-4 = CLN3906		n.t	n.t	R	S	n.t	H	S	n.t	n.t
B5			n.t	n.t	H	S	n.t	R	S	n.t	n.t

R: Resistance allele, homozygote, S: Susceptibility allele, homozygote, H: Heterozygote, 3a: Carrying the resistance allele Ty-3a, n.t: Not tested

the *Ty-2* locus, as well as for the root-knot nematode resistance gene, publically available SCAR markers were used to determine the genotype of tomato breeding material at these loci (Garcia *et al*., 2007; Shirasawa *et al*., 2010; Seah *et al*., 2007) (Table 2).

Polymerase chain reactions for single marker assays were performed in 10 µL reactions containing 0.25 µM of forward and reverse primers, 0.2 µM of deoxyribonucleotides, 50 mM KCl, 10 mM Tris HCl (pH 8.3), 1.5 mM $MgCl_2$, 25 ng of DNA and 0.5 U of Super-Therm Gold DNA polymerase (Bertech Enterprise Co., Ltd.). The amplification profile was initial denaturation at 95°C for 10 min, followed by 35 cycles of 94°C for 45 sec, primer specific annealing temperature for

Table 2: Molecular marker tested or used for the detection of tomato yellow leaf curl and nematode resistance loci

R-gene	Marker	Type	Chr.	Pos.	Forward primer	Reverse primer	Fragment size	References
Ty-1	TG178	SCAR	6	21.040	GAGTCCCTAACGAATGGTCCTACT	GCAGACAAATGCTCAAAGGTCACACC	Multiple bands	Barbieri *et al*. (2010)
Ty-3	P6-25	SCAR	6	31.499	GGTAGTGGAAATGATGCTGCTC	GCTCTGCCTATTGTCCCATATATAACC	~450/319	Ji *et al*. (2008)
Ty-1/3	M2	SCAR	6	30.879	GATCCGTTGATTGAAGAAAT	AGGAAGAGGAGAGACAATCC	264/252	Present study
Ty-1/3	TY-1/3_K	SCAR	6	30.879	ACAGGAAAAATGGGTGATCC	CCTGCTCCTTGCAGATTCTA	114/102	Present study
Ty-2	T0302	SCAR	11	51.0878	TGGCTCATCCTGAAGCTGATAGCGC	AGTGTACATCCTTGCCATTGACT	~900/791	Garcia *et al*. (2007)
Ty-2	TES0344	SSR	11	51.420	GCCTTTTCCCACTTATATTCCTCTC	ACACATACGACGTTCCGTCA	190/205	Yang *et al*. (2012)
Ty-5	AVRDC-TM719	SSR	4	1.450	TCGATTTGGAATGAGTTTTC	TGAAATAGATTTGTCAGGTGTT	-/237*	Present study
Ty-5	SLM4-34	SSR	4	2.938	GACCATTAACCTCGATCA	GAAAGTCATGTGAATAGCAG	Multiple bands	Kadirvel *et al*. (2013)
Ty-5	SINAC1 (TAQ I)	SSR	4	2.856	TGCCTGGTTTCTGCTGTCA	TAAAGCTGAAGAAGGACTTACCCT	Multiple bands	Anbinder *et al*. (2009)
Ty-5	AVRDC-TM273	SSR	4	3.200	GGTGCTCATGGATAGCTTAC	CTATATAGGCGATAGCACCAC	~180/173	Present study
Ty-5	AVRDC-TM81	SSR	4	5.990	GTATGGAGAGTCGAGTCCTG	CCATGATAAGTAGCGAGAGG	-/153*	Present study
Ty-5	AVRDC-TM70	SSR	4	7.340	TTTCTTTGTTTCCTTTCAGTG	GCCTTGGACAAGGTACAATA	Multiple bands	Present study
Ty-5	AVRDC-TM947	SSR	4	7.340	TGCGTCTAGTTTTCTTTGTTT	CAAGCTGAAAGGAATTCAAC	Multiple bands	Present study
Mi-1	PM3F/R	SCAR	6	2.722	CCTGTGATGAGATTCCTCTTAG	ACCCTTTGTTGAGCGACTTTGCAGC	~610/592	El Mehrach *et al*. (2005)
Mi-1.2	Mi23	SCAR	6	2.322	TGGAAAAATGTTGAATTTCTTTTG	GCATACTATATGGCTTGTTTACCC	~380/431	Seah *et al*. (2007)

Fragment size for the susceptibility (S) alleles and the approximate positions of the markers were determined based on the SL2.40 tomato whole genome sequence (The Tomato Genome Consortium, 2012), Length of the resistance (R) allele bands were estimated after agarose or polyacrylamide gel electrophoresis using 50 bp DNA size markers, *Monomorphic markers. R-Gene: Resistance gene name. Chr.: Chromosome, Pos.: Position of chromosome in mega base pairs according to the tomato reference genome, Fragment size: Fragment sizes of the band for the resistant and for the susceptible genotype

1 min, 72°C for 2 min and final extension for 10 min at 72°C. The PCR products (2 µL) were size-fractionated, depending on the fragment size, either on 1.5% agarose gels, or for fragments smaller than 300 bp, on 6% non-denaturing polyacrylamide gels in 0.5×TBE buffer. After electrophoresis, the gels were stained with ethidium bromide and DNA bands were visualized under ultraviolet light.

Verification of the *Ty* and *Mi-1* genotype in tomato breeding lines: Tomato breeding lines, the two wild accessions and the potentially heterozygous F_2 breeding material was verified for their genotypes at the *Ty-1, Ty-2, Ty-3, Ty-1/3, Ty-5* and *Mi-1.2* loci using individual marker assays (Table 1). From the two F_2 families, four and five individuals, respectively that contained homozygote and heterozygote resistant and susceptible genotypes at the four different loci were selected for testing the multiplex assay. The PCR conditions for marker genotype verification were the same as outlined above for primer design for PCR-based *Ty-1/3* and *Ty-5* markers.

Multiplex PCR assay: For the final version of the multiplex PCR test, forward and reverse primers for markers M2, T0302, AVRDC-TM273 and Mi23 (Table 2) were combined in a concentration of 0.15 µM each. The amount of deoxyribonucleotides in the multiplex assay was increased to 0.25 µM and 4% non-denaturing polyacrylamide gels were used to resolve the wider size range of PCR products ranging from 173 to about 900 bp.

Replacing single markers of the multiplex assay: Marker Mi23 was replaced either with marker R2M1S2 associated with resistance of tomato to *Phytophthora infestans* (Zhang *et al*., 2014), or SLM12-2 linked to bacterial wilt resistance (Wang *et al*., 2013). To accommodate SLM12-2 in the multiplex assay, marker M2 detecting the *Ty-1/3* resistance locus was redesigned based on the sequences given in Verlaan *et al*. (2013) to give a shorter fragment of 100 bp to ensure appropriate spacing of the different marker bands. The re-designed marker was termed TY-1/3_K.

RESULTS

The genotypes of the 18 tomato breeding lines were verified at the four tomato yellow leaf curl and the one root-knot nematode resistance loci using PCR-based markers, which are routinely used for marker-assisted selection at AVRDC-The World Vegetable Center (Table 1, 2). Additionally, a newly designed SCAR marker targeting the 12 base pair deletion in the susceptibility allele of the *Ty-1/3* locus was tested. The *Ty-1/3* marker discriminated only between resistance and susceptibility alleles at the *Ty-1/3* locus, but not between the *Ty-1, Ty-3* or *Ty-3a* resistance alleles. The newly designed Ty-1/3 marker reliably indicated resistance in plants homozygous for *Ty-1*, *Ty-3* or for the *Ty-3a* allele, in single marker assays (Table 1). Both the SCAR marker T0302 and the SSR marker TES0344 detected the resistance and susceptibility alleles at the *Ty-2* locus of the homozygous plants, but yielded an aberrant banding pattern in *S. chilense* accession LA1932, indicating that this *Ty-2* susceptible wild accession contains different a *Ty-2* allele than *S. lycopersicum*. The *Ty-5* resistance locus usually is detected by the CAPS marker SINAC1. The CAPS markers are not practical for multiplex PCR assays, therefore a new diagnostic marker for the *Ty-5* resistance allele was needed. Six SSR markers located on chromosome 4 between position 1.45 and 7.34 Mb and surrounding marker SINAC1 (at position 2.856 Mb) were screened for correct prediction of the *Ty-5* genotype in the breeding line panel. It showed that two out of the six SSR markers were polymorphic between plants carrying the *Ty-5* resistance and susceptibility alleles.

One of these markers, SLM4-34, produced several PCR fragments of multiple sizes, while AVRDC-TM273 yielded a single PCR product specific for the *Ty-5* resistant and susceptible genotype in the homozygote breeding material. In *Ty-5* susceptible *S. chilense,* AVRDC-TM273 yielded different banding patterns than in *S. lycopersicum*, suggesting the presence of different alleles at this locus in this species than in cultivated tomato. Both tested markers for *Mi-1* amplified gave easily scorable DNA bands from the breeding material and the two wild accessions and identified correctly tomato line 132116 carrying the *Mi-1* resistance gene (Table 1, 2).

Based on their diagnostic capacity, five SCAR and two SSR markers became candidates for a multiplex PCR assay testing three tomato yellow leaf curl and one root-knot nematode resistance loci (Table 2). For the multiplex assay, PCR markers were chosen that gave simple band patterns and showed clear size differences between the bands, indicating the susceptibility and resistance alleles. Additionally, the chosen markers had to yield PCR fragments of different size categories for each of the resistance loci in order to resolve the fragments derived from four different loci on a gel. Based on these parameters, the markers T0302 (*Ty-2*), Mi23 (*Mi-1*), M2 (*Ty-1/3*) and AVRDC TM273 (*Ty-5*) were chosen. Their PCR fragments ranged in size between 173 to about 900 bp. The multiplex PCR was first tested on the 18 breeding line set and the two wild *S. chilense* accessions. The primer concentration for each of the markers was lowered to a final concentration of 0.15 µM. After obtaining weak amplification, the dNTP concentration in the assay was increased to 0.25 µM. In order to resolve all bands in the size range from 173-900 bp and also visualize small size differences among the smaller bands, the polyacrylamide concentration in the gel was reduced from 6-4%.

The resultant multiplex PCR assay successfully amplified DNA fragments diagnostic for the *Ty* and *Mi-1* genotype from all homozygote breeding lines and the genotyping results of the multiplex assay were consistent with the results obtained with individual tests at the four loci (Fig. 1). The PCR fragments of each of the markers were well separated from each other, allowing for clear scoring of the susceptibility or resistance allele at each locus. As with the single marker PCR reactions, the multiplex assay also gave different bands for the susceptibility allele at the *Ty-5* locus in both *S. chilense* accessions and for *Ty-2* in *S. chilense* CLN1932. However, the resistance at *Ty-1/3* and susceptibility at *Mi-1* in the two *S. chilense* accessions was correctly shown by the assay (Fig. 1a).

In potentially heterozygote F_2 plants, the multiplex PCR test correctly predicted homo-or heterozygote presence of resistance and susceptibility alleles at all four loci (Fig. 1b). However, *Mi-1* and *Ty-5* heterozygote genotypes produced hybrid DNA fragments derived from the two alleles, which migrated with the *Ty-2* and *Ty-1/3* bands, respectively. These bands derived from the hybrid DNA fragments were not specific for the multiplex assay and were also produced in single marker tests (Fig. 2). Despite these additional bands, homo-and heterozygote *Ty-1/3* and *Ty-2* resistant and susceptible plants could be identified.

To test whether the multiplex assay can be extended to detect resistance alleles at other loci, the Mi23 marker detecting root-knot nematode resistance was replaced either with marker R2M1S2 or SLM12-2, which are routinely used to select for resistance of tomato against *Phytophthora infestans* and *Ralstonia solanacearum* in single marker assays (Zhang *et al*., 2014; Wang *et al*., 2013). Replacing one marker did not affect the diagnostic capacity of the multiplex assay (Fig. 3), although the introduction of marker SLM12-2 caused the production of two bands instead of one for the susceptibility allele of *Ty-2*.

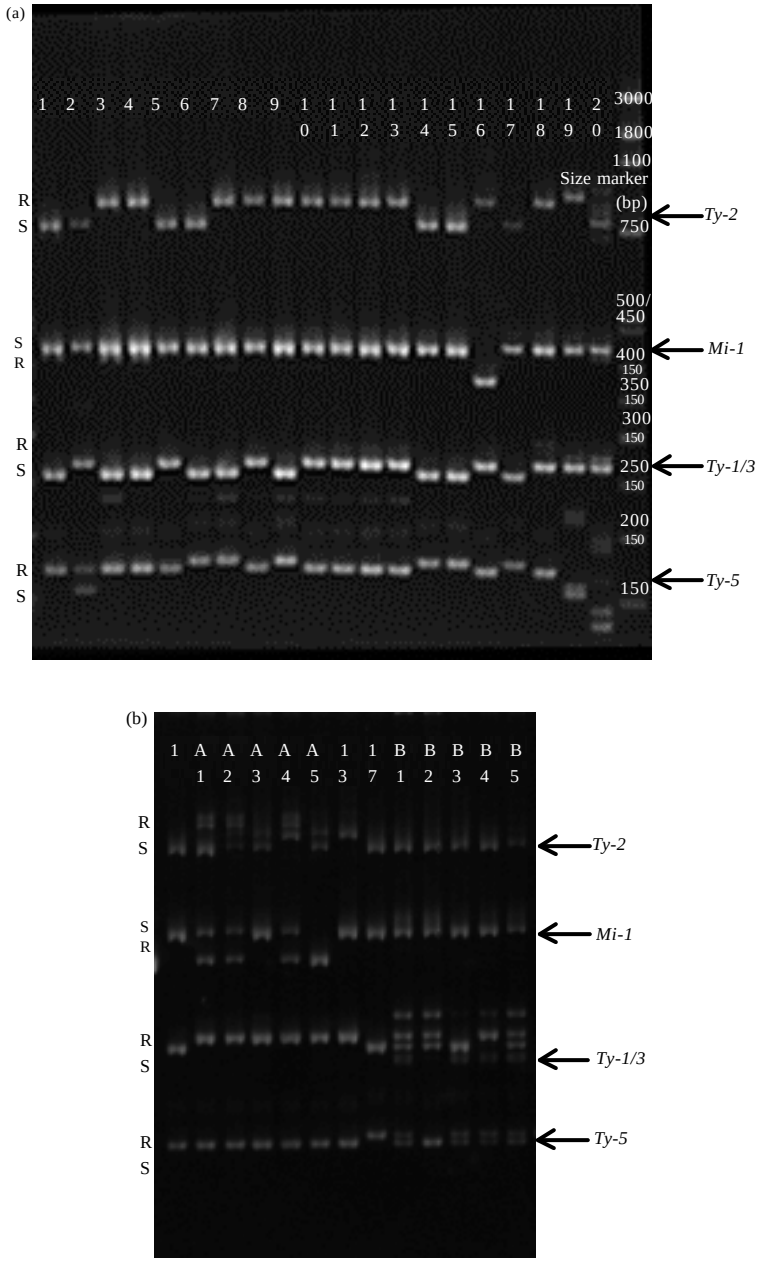

Fig. 1(a-b): Multiplex Polymerase Chain Reaction (PCR) detection of (a) *Ty-1/3*, *Ty-2*, *Ty-5* and *Mi-1* alleles in breeding lines and (b) In potentially heterozygote material. The numbers on top of the bands correspond to the ID numbers of the plant materials, 1: Tanya, 2: Ty52, 3: CLN2498D, 4: CLN3024A, 5: CLN3205B, 6: CLN3212C, 7: CLN3150A-5, 8: CLN3126A-7, 9: CLN3447G, 10: CLN3070J, 11: CLN3241H-27, 12: CLN3125P, 13: CLN2819B, 14: F8-48, 15: F9-159, 16: CLN3682F1-10-3-4-27-1, 17: FLA456, 18: CLN3125K, 19: LA1969, 20: LA1932, A1 to A5: Different heterozygote F1 individuals of cross CLN3900, B1 to B5: Heterozygote F1 individuals of cross CLN3906. R and S indicate the position of the bands for the resistance and susceptibility alleles

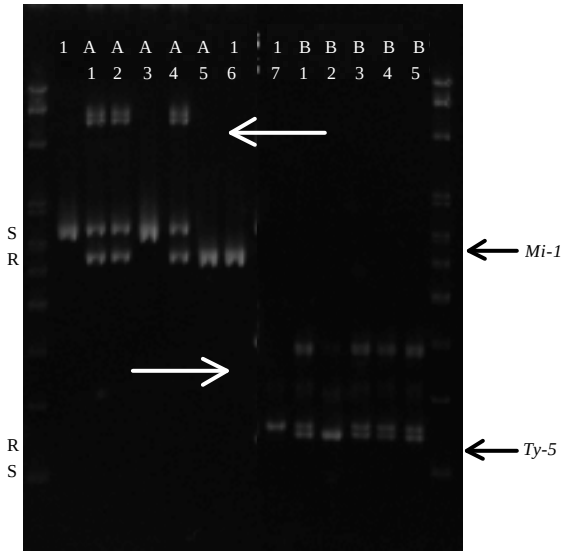

Fig. 2: Single marker PCR for *Mi-1* (1, A1 to A5, 16) and *Ty-5* (17, B1 to B5). In heterozygote plants, additional to the diagnostic band (marked with R and S), larger bands appear (marked with white arrows), which are located near the *Ty-2* and *Ty-1/3* bands of the multiplex assay (Fig. 1b). The test plants used were 1: Tanya, A1 to A5: Different heterozygote F1 individuals of cross CLN3900, 16: CLN3682F1-10-3-4-27-1, 17: FLA456, B1 to B5: Heterozygote F1 individuals of cross CLN3906

These results demonstrated that multiplex PCR can be applied for reliable screening of tomato breeding lines for the homo or heterozygous presence of resistance or susceptibility alleles at four genetic loci simultaneously. The application of the test on wild relatives of tomato such as *S. chilense* is limited by the presence of "wild" alleles in this material.

DISCUSSION

Tomato yellow leaf curl and root-knot nematodes are important constraints of tomato production worldwide. Many tomato breeding programs aim to include resistance genes against these diseases into varieties. Marker-assisted selection is a cost-saving method to introgress and manipulate resistance genes in breeding populations (Slater *et al*., 2013). In the case of recessive resistance genes, selection through molecular markers provides the advantage of easy distinction between heterozygote and homozygote susceptible lines, making the use of recessive resistance genes such as *Ty-5* in breeding easier. Pyramiding multiple resistance genes against a disease such as tomato yellow leaf curl is difficult to achieve by classical breeding. Breeders using marker-assisted selection can combine multiple resistance genes against tomato yellow leaf curl in a line, which is likely to extend resistance against multiple viral strains. For example, *Ty-2* alone provided strong resistance to the monopartite *Tomato leaf curl Taiwan virus*, but not against bipartite *Tomato yellow leaf curl Thailand virus*. Combining *Ty-2* with *Ty-1*, *Ty-3* or *Ty-3a* is expected to provide better resistance against both begomoviruses. Stacking multiple resistance genes is most efficiently accomplished by marker-assisted selection. The described multiplex PCR method reliably detects three important tomato yellow leaf curl resistance loci from the wild tomato species

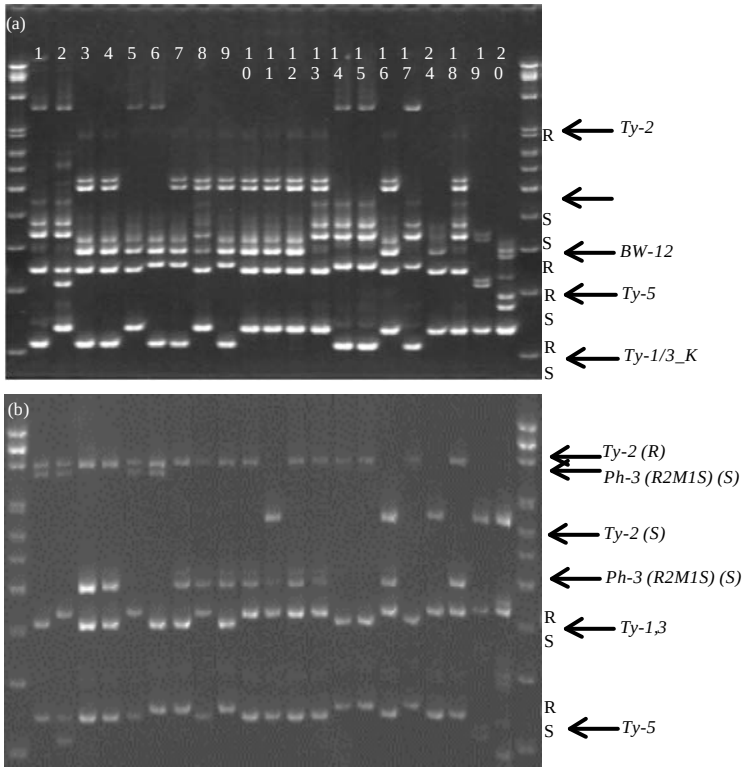

Fig. 3(a-b): Replacement of marker M23 (*Mi-1*) with (a) R2M1S2 and (b) SLM12-2. The numbers on top of the bands correspond to the ID numbers of the plant material, 1: Tanya, 2: Ty-52, 3: CLN2498D, 4: CLN3024A, 5: CLN3205B, 6: CLN3212C, 7: CLN3150A-5, 8: CLN3126A-7, 9: CLN3447G, 10: CLN3070J, 11: CLN3241H-27, 12: CLN3125P, 13: CLN2819B, 14: F8-48, 15: F9-159, 16: CLN3682F1-10-3-4-27-1, 17: FLA456, 18: CLN3125K, 19: LA1969, 20: LA1932, 24: T-21. R and S indicate the position of the bands for the resistance and susceptibility alleles

S. chilense, S. habrochaites and *S. peruvianum* and thus facilitates marker-assisted resistance gene pyramiding. Previously, multiplex PCR tests for tomato resistance gene alleles have been described, but these tests either targeted a smaller number of loci (Chen *et al*., 2012; Fu *et al*., 2013) or a different locus combination (Liu *et al*., 2013).

This method showed that the susceptibility alleles at two *Ty* resistance gene loci in *S. chilense* accessions were different than those in *S. lycopersicum*. Consequently, single target as well as multiplex PCR gave different banding patterns for these alleles for wild and cultivated species. Additional bands were observed for *Mi-1* and *Ty-5* in heterozygote plants, in both single marker and multiplex assays. These additional bands probably are produced by heteroduplexes formed between amplification products of different sizes in heterozygous genotypes. However, the additional bands did not affect the correct prediction of the marker genotypes.

CONCLUSION

Combining multiple PCR tests into a multiplex assay decreases working costs and makes marker-assisted selection more affordable for breeding programs. The present assay combines PCR

tests detecting four commonly used resistance loci of tomato against economically important diseases in the time and with the reagents (except primers) of one test, thereby reducing genotyping costs and workload by about 75%. Since the multiplex PCR assay was tested on a representative panel of breeding lines, on selected individuals of two F_2 populations and on two wild tomato accessions, it is assumed that the assay reliably detects the four resistance alleles in a broad range of breeding materials. Single markers of the multiplex assay could be replaced by others, allowing breeders to adapt the assay to the specific needs of the breeding program.

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