

Markers for Rapid Evaluation of Virus Resistance for TYLCV in Tomato, ZYMV and PRSV-W in Zucchini and LMV in Lettuce and Hybrid Seeds in Pumpkin

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Abstract

Screening for the source of virus resistance in horticultural plants or specific characterization as hybridization, through symptoms, requires time and depends on the weather and knowledge of plant characteristics. So, it is important to develop specific gene markers to allow rapid diagnosis by PCR. Markers were developed based on sequences homology comparison of susceptible and resistant plants provided by HORTEC SEEDS in tomato for *Tomato yellow leaf curl virus* (TYLCV) by the resistance gene *Ty-1*, in zucchini for *Zucchini yellow mosaic virus* (ZYMV) and *Papaya ringspot viruses* tirpe watermelon (PRSV-W), and in lettuce for *Lettuce mosaic virus* (LMV). Fragments of 249 bp were amplified only by resistant plants to TYLCV as the hybrids 2648 and Aguamiel, and not for varieties as Santa Cruz or Carina. It were observed for ZYMV the amplification of 791 bp by the resistant hybrid Px7051 and not for the susceptible cultivar La Belle; for PRSV-W using the same zucchini plants the amplification of 650 bp for susceptible and 750 bp for resistant; for LMV the 421 bp amplification only for the resistant cultivar Brasil 303 and not for susceptible Babá de Verão. Finally, it was observed that primers PK47F/R were able to check the Cabotiá seed hybrids of pumpkin Jabras.

Keywords

Tomato Yellow Leaf Curl Virus, Zucchini Yellow Mosaic Virus, Papaya Ringspot Virus Strain Watermelon, Lettuce Mosaic Virus, Jabras Hybridization

1. Introduction

Since the major diseases of horticultural plants as tomato (*Solanum lycopersicum* L.), zucchini (*Cucurbita pepo* L.) and lettuce (*Lactuca sativa* L.) are caused by virus, whose high biological diversity demands resistant material as the best way of disease controlling, the development of specific primers for identification of resistance genes will allow the resistance source screening by a common PCR, in order to facilitate the incorporation of desirable genes.

The begomovirus *Tomato yellow leaf curl disease* (TYLCD) is one of the main limiting factors for tomato production [1], which belongs to the monopartite *Tomato yellow leaf curl virus* (TYLCV). As the most widespread resistance used commercially is based on the *Ty-1* resistance gene, specific markers were developed for this gene. CAPS (cleaved amplified polymorphic sequence) markers (requiring restriction enzymes activity), such as Aps-1 or Rex-1 (both linked to the nematode resistance *Mi* gene, giving false positive results because of joint introgression) were replaced by others as JB-1 [2]. The region of JB-1 marker has been reported not hybridized with *S. lycopersicum* along with the *Mi* gene, so the presence of this gene does not interfere with marker [2]. The accessions and inbred lines that carry the *Ty-1* gene present high levels of resistance for monopartite (TYLCV) or bipartite types such as *Tomato severe rugose virus* (ToSRV); thus the SCAR (Sequence Characterized Amplified Region) marker that does not need restriction by enzyme, called C-19, was developed by the ToSRV evaluation in tomato [3].

In Brazil the main diseases in Caserta or Italian zucchini (*Cucurbita pepo* L.) are caused by potyviruses such as *Zucchini yellow mosaic potyvirus* (ZYMV) or *Papaya ringspot virus strain watermelon* (PRSV-W). Although ZYMV resistance genes have already been reported in *Cucurbita moschata* L., consistent molecular markers have been reported for watermelon, melon and cucumber [4]. For zucchini and squash, CAPS [5] and SCAR types have been reported as, without detailing the marker [6], or with few bases of difference (which hinders specific identification) [7], or about 0.8 cM of the *Zymv* locus (originated from the RAPD UBC522-TCGTCTAGCA) called SCAR SC522₉₄₅ which, however, showed inconsistent inoculation and backcrossing results [8]. Two SSR markers were identified respectively for the *Zym-1* and *Zym-0* genes [9].

Regarding marker for resistance to PRSV-W in pumpkins, only RG-like sequences (SQRGA1 to 6, SQRGA8 to 14), with accesses in GenBank EF101660 to EF101665, EF101667, EF199755 to EF199760 were reported [10]. In melon, two CAPS-like markers for the dominant *Prv1* gene (one RGH NB-LRR with the sequence AF354504 and one RAPD with sequence AY611532) were reported [11], whereas the AFLP (EK 190) with a 150 bp fragment bound to the *Prv1* resistance gene reported by another author [12]. In cucumber the RAPD marker AP7-1800 was reported [13] while the CN patent No. 104313017A dated 02/28/2015 reported the indel marker (insertion or deletion of bases) for the PRSV resistance gene (prsvIndel2-F/prsvvIndel2-R), which amplified, 176 bp (SeqIDNo1 sequence)

and 170 bp (SeqIdNo2 sequence), respectively, for susceptible and resistant genotype.

The *Lettuce mosaic virus* (LMV) recessive resistance gene called *mo-1* has 2 alleles that encode translation initiation factor (eIF4E). By the central region sequencing of eIF4E cDNAs from tolerant, susceptible and resistant lettuce genotypes to LMV (GenBank AF530162), three types of alleles, Ls-eIF4E¹, Ls-eIF4E² and Ls-eIF4E[°] were found and using RT-PCR was reported the eIF4E-PagI CAPS marker (generated with PagI restriction of Ls-eIF4E cDNA) [14]. A complete correlation between Ls-eIF4E¹ and the *mol*¹ gene, Ls-eIF4E² and the *mol*² gene, Ls-eIF4E[°] and susceptible genotype were reported [14].

Marker is also important for genetic characterization. One of the main quality characteristics of seed marketing is genetic purity, especially of hybrid seeds, since self-pollination of the female parent is a major cause of contamination. Purity is important to leverage the distribution of national (cheaper than imported) hybrid seeds of Cabotiá pumpkin to ensure uniformity and stability of production. As morphological criteria can be influenced by environmental conditions and require a high degree of knowledge for phenotypic identification, molecular methodologies are highly desirable. RAPD [15] and microsatellites [16] [17] [18] have been reported, however, low reproducibility has been reported using RAPD [19] and separation of very small microsatellites fragments requires expensive apparatus or laborious electrophoresis.

Therefore, specific marker that allows the knowledge of the desirable genotype with a simple PCR reaction is important to aid the improvement work of horticultural plants such as tomato, zucchini, pumpkin and lettuce.

2. Materials and Methods

2.1. Materials

2.1.1. For Tomato

Young tomato leaves supplied by HORTEC SEEDS of TYLCV resistant hybrids such as Aguamiel (VILMORIN do BRASIL), 2648 (HORTEC SEEDS) and susceptible varieties such as Carina (SAKATA), Santa Cruz and Santa Clara were used. In addition, first 90 and then 75 resistant and susceptible tomato accessions (HORTEC SEEDS, lineages and hybrids) were screened for the presence of resistance genes. To compare the marker developed for detection of the *Ty-1* resistance gene, the primers for To SRV resistance [3] (Table 1) were used, which amplify fragments of approximately 800 bp for susceptible plant, fragment slightly smaller for resistant plant and both types of fragments for heterozygotes.

2.1.2. For Zucchini

Young leaves of the resistant hybrid Px7051 from SEMINIS and the susceptible cultivar La Belle from HORTEC SEEDS were used, as well as a hundred and ten accessions (lineages and hybrids) for screening the resistance to ZYMV and PRSV.

Table 1. Sequences of primers developed for virus resistance or hybrid characterization in plants of tomato, zucchini and lettuce, where T_m = melting temperature in °C; L = amplification length in bp; S = susceptible and R = resistant; T = annealing temperature in °C; A = Author of the developed primers.

Event	Sequences (5'-3')	T _m	L	T	A
TYLCV (<i>Ty-1</i>) resistance	562 JB: AAG AGT TAG CTA GAT ATT C	50	269	46	From this work
	830 JB: ACC TTT TGT ACG TAG AT	51			
ZYMV resistance	36 F: GAC TAC GCC TTC AAG ACT CA	65	790	52	From this work
	826 R: CTG TCC AAC CAT TTC TAG GG	67			
PRSV resistance	408: CGG TCA ACG CAA TTC TGT	68	S-650 R-750	44	From this work
	552: TTA ATA TAC TTT AAA CAT	44			
LMV resistance	228 FRes: CCT AAG TCC AAG CAA GTC	60	421	46	From this work
	648 RSusc: AAT CGT ATG GTT CAT TGC	61			
ToSRV (<i>Ty-1</i>) resistance	SCARC19F: GTT GCC AGC CCC ATG AGA TTT T	63	S = 800 R < 800	60	[3]
	SCARC19R: GTT GCC AGC CGA GGC CAA GTT T	66			
HybridJabras	PKCT47F: GGT CCC AAT AAT AGC AAC CAA			46	[18]
	PKCT47R: GTG GGA CAC ATC TTG AGC A				

2.1.3. For Lettuce

Young leaves of the cultivars, Babá de Verão (susceptible) and Brazil 303 (resistant) besides the resistant accessions 1019 and 1719 provided by HORTEC SEEDS.

2.1.4. For Hybrid Seeds Characterization

Seeds and leaves of the maternal (1,405,012 or 145,083) and paternal (135,122 or 145,084) progenitors and the respective hybrid of the Cabotiá pumpkin named Jabras (*C. maxima* X *C. moschata*) provided by HORTEC SEEDS.

2.2. DNA Extraction

DNA extraction from leaves was done using a modified CTAB methodology [20] and seeds by another CTAB method [21].

2.3. PCR Reactions

All PCR reactions were done in a volume of 15 µL with 10 µL of Mix and 5 µL of DNA diluted to 20 - 50 ng according to the event, with initial denaturation at 95°C for 1 minute and 30/35 cycles. The electrophoresis was performed with 1% or 1.5% agarose gel.

1) Reaction for *Ty-1* gene of TYLCV resistance in tomato using the primers 562 JB/830JB with amplification of the 269 bp fragment by resistant genotype was performed with 20 ng DNA, 2 mM MgCl₂, 0.167 mM dNTP, 10 µM of each

primer, 1.25 units of Taq enzyme (INVITROGEN) and 30 cycles of 20 s at 95°C, 25 s at 44°C and 20 s at 72°C. The PCR reaction using primers for ToSRV resistance [3] was performed according to the author's conditions, but using 1.25 units of Taq (INVITROGEN) and 1 mM of each primer.

2) Reaction for ZYMV resistance in zucchini using primers 36 ZYM/826ZYM with amplification of 791 bp fragment by resistant genotype was performed with 30 ng DNA, 2 mM MgCl₂, 0.167 mM dNTP, 10 µM of each primer, 1.25 units of Taq enzyme (INVITROGEN) and 30 cycles of 25 s at 95°C, 40 s at 52°C and 50 s at 72°C.

3) Reaction for PRSV resistance in zucchini using primers 408 PRS/552PRS with amplification of 650 bp and 750 bp fragments, respectively by susceptible and resistant genotypes was performed with 30 ng DNA, 2 mM MgCl₂, 0.2 mM dNTP, 10 µM of each primer, 1.25 units of Taq enzyme (INVITROGEN) and 30 cycles of 25 s at 95°C, 40 s at 44°C and 45 s at 72°C.

4) Reaction for LMV resistance in lettuce using primers 228 R/648S with amplification of 421 bp fragment by resistant genotype was performed with 50 ng DNA 2 mM MgCl₂, 0.167 mM dNTP, 10 µM of each primer and 1.25 units of Taq enzyme (INVITROGEN) with 30 cycles of 25 s at 95°C, 60 s at 46°C and 30 s at 72°C.

5) Reaction for diagnosis of the Jabras hybrid using primers PKCT47F/PKCT47R [18] was performed with 30 ng DNA, 2 mM MgCl₂, 0.2 mM dNTP, 10 µM of each primer, 1.25 units of Taq enzyme with 35 cycles of 25 s at 95°C, 40 s at 46°C and 30 s at 72°C.

2.4. Primers Development

The primers developed for each marker were listed in **Table 1**. Sequence alignment for homology comparison was performed using BIOEDIT [22]. Sequencing was performed by LACTAD at UNICAMP in the city of Campinas, Brazil. Primer 3 program is for design the primers.

1) For *Ty-1* gene: Using the primers reported as strongly linked to the *Ty-1* gene, termed JB-1F/JB-1R [2], fragments of 930-bp amplified by the genomes of *S. lycopersicum*, *S. chilense* and *S. peruvianum*, were reported [23], which sequences showed the same indels of the *Ty-1* gene associated with the resistant chilense line LA1969S, but with characteristic regions. In order to develop marker for the *Ty-1* gene, we compared the three 930 bp reported sequences [23].

2) For resistance to ZYMV, 38 primers resulting from the comparison of sequences of *C. moschata* with watermelon RGAs [4], or from RGAs and Unigenes of *C. pepo* [24] [25], or by sequencing of marker amplicons reported in pumpkin close to the resistance gene, such as the SCAR SC522₉₄₅ [8].

3) To characterize the presence of the *PRSV* or *prsv* genes linked to the PRSV resistance, 19 primers resulting from the comparison of the resulting sequences: from CAPS markers to the dominant *Prv¹* gene in the melon, such as primers 408 PRSV/1155PRSV [11]; of RAPD AP7-1800 (5'-GTG GAT GCG

A-3') bound to the recessive gene [13]; resulting from the patent CN 104,313,017 A which reports an indel in the region of PRSV resistance gene in cucumber (prsvIndel2-F/prsvvIndel2-R) with sequences SeqID No1 and SeqID No1 which, after BLAST, resulted in the melon sequences LN713265 and LN68192, yielding through the indel region of cucumber, the primers (such as 243 PRSV/552PRSV); by comparing the eIF4E and eIF (iso) 4E genomic regions of cucumber and melon associated with recessive inheritance of viral resistance [26].

4) Using the same cDNA sequences obtained by RT-PCR reaction with the CAPS eIF4E-PagI marker, from resistant genotypes like Salinas 88 and susceptible as Salinas (GenBank accession numbers AF530162-4E and AF530163-4E) reported [14], and using the SAP-PCR Methodology (Single allele discriminating PCR) [27], the primers were developed to differentiate susceptibility and resistance. The SAP-PCR method requires two PCR reactions, with two sets of primers (for susceptibility and for resistance), since both amplify fragment of the same size. A forward primer and two reverse primers (one for susceptibility and another for resistance) were developed by the SAP-PCR method, with the addition of different combinations of bases at the last sites to destabilize the base pairing between susceptible primer and resistant genome, or vice versa.

5) The polymorphism between progenitors was tested using the RAPD reported in *C. maxima* [15] and analyzing SSR markers from the literature that allow the amplification of large fragments. Twenty two SSR primers developed for pumpkins [16] [18] or zucchini (Applied Life Sciences the Institute of Biotechnology in Plant Production, Department for Agrobiotechnology of the BOKU-University of Natural Resources, Austria) were tested.

3. Results and Discussion

For resistance to TYLCV, the primer set 562 JB/830JB developed for the *Ty-1* resistance gene (Table 1) amplified a distinct band of 249 bp only for resistant plants. The sequence of this fragment showed similarity of 100% with the corresponding sequence reported [23], giving 99% resemblance with *S. chillense*, 84% for *S. peruvianum*, 94% for *S. lycopersicum* and 93% for chromosome 06 of *S. lycopersicum* (Genbank HG975518). The analysis of a hundred and sixty five tomato accessions (HORTEC SEEDS) gave a positive result of 25% for the *Ty-1* resistance gene. Figure 1(a) shows the PCR amplification profile using primers 562 JB/830JB for the two resistant hybrids and three susceptible cultivars, where 2648 and Aguamiel amplified the 249 bp fragment, whereas Carina, Santa Cruz and Santa Clara did not. Using the primers for resistance to ToSRV [3] and almost the same conditions as the author to analyze resistance to the *Ty-1* gene, the same results reported for amplification of an 800 bp fragment per susceptible plant, slightly smaller fragment per resistant plant, and both types of fragments by heterozygotes, were verified (Figure 1(b)), but the electrophoresis of at least seven hours needed to separate the 800 bp amplification and allow the differen-

tiation between resistant and susceptible was very laborious and time consuming. The specific primers 562 JB/830JB developed in this work verified the characteristic regions previously reported [23], which made possible the amplification of a polymorphic fragment. Furthermore, the 93% similarity of the primer amplicon sequence developed with *S. lycopersicum* chromosome 06 strengthened its closeness to the *Ty-1* resistance gene located on the same chromosome. A multiplex PCR using CAPS primers for the JB-1 and Mi genes identified at the same time the genotypes of resistance to the root-knot nematode and TYLCV in the F1 and F2 generations [28]. So, the development of a marker derived from JB-1, which does not require enzymatic digestion, will make easier the screening of TYLCV resistance and incorporation of this allele by marker-assisted selection.

Among the 38 primers tested for ZYMV resistance, the best was originated from SCAR SC522₉₄₅ [8], which provided the GenBank sequence MG888015 used for the development of the primer set 36 R/826 R (Table 1), which amplified the fragment of 791 bp only for the resistant hybrid Px7051 and not for the susceptible cultivar La Belle, as shown in Figure 2(a), where three DNA concentrations (20, 30, 40 ng) showed no differences. The results of the analysis performed with 110 zucchini samples from HORTEC SEEDS as showed for thirteen samples in Figure 2(b) were confirmed with those obtained by mechanical inoculation of the ZYMV, by the researcher Valdir Atsushi Yuki of the IAC Plant Protection Center. So, the development of a specific marker for zucchini ZYMV resistance, will become easier the screening and incorporation of resistance by marker-assisted selection.

From the 19 primers tested for PRSV-W, the best primer set was formed with forward primer 408 PRSV, which was originated by the comparison of sequences of CAPS markers for the dominant *Prv¹* gene [11], and with the reverse primer 552 PRSV, originating from melon sequences as LN713265 and LN68192 (which resulted from the comparison of sequences in the indel region of the *prsv* resistance gene in cucumber provided by the patent CN 104,313,017) giving the set of primers 408 PRSV/552PRSV (Table 1) that differentiated the resistant hybrid Px7051 and the susceptible cultivar La Belle. The polymorphism was observed by two fragments with about 650 bp for the susceptible sample and 750 bp for the resistant one, plus other nonspecific bands. Figure 3 shows the PCR reaction profile using the 408 PRSV/508PRSV primers, for sixteen samples and two of each control for resistance (Px7051) and susceptibility (La Belle), where it is possible to characterize ten samples as resistant and six as susceptible. Of the 110 zucchini samples provided by HORTEC SEEDS that showed 85% resistance to ZYMV, about 78% presented resistance to PRSV-W. Although most of the ZYMV-resistant samples also showed the gene for resistance to PRSV-W, not every ZYMV resistant sample showed the PRSV-W resistance gene, with 92% agreement between the two data. These results suggest the specificity of this marker for resistance to PRSV-W in zucchini, facilitating the screening and incorporation of resistance by marker-assisted selection.

For resistance to LMV, the reactions using the forward primer, located in the SNP at base 228 of the resistant genome, and reverse primers of the susceptible and resistant genomes, which were developed around SNP at base 632 (with base changes according to the SAP-PCR), showed polymorphic fragments. The 228 Res/649Res primer set amplified a fragment larger than the expected (420 bp) by the resistant genome, while the 228 R/648Susc set (**Table 1**) amplified a fragment of about 420 bp but also by a resistant rather than susceptible genome. Therefore, with only one PCR reaction it was possible to characterize the resistant genome. **Figure 4(a)** shows the base pairing in the region used for the development of 648 Susceptible and 649 Resistant reverse primers, showing bases with SNIP bases or changes in bold and larger. The 228 Res/648 Susc set had better performance as it amplified a more easily visible fragment of about 420 bp, as shown in **Figure 4(b)** by the Brazil 303 resistant lettuce cultivar with two different concentrations (40 and 50 ng) and the resistant accessions 1019 and 1719, while the susceptible cultivar Babá de Verão with the same concentrations did not amplify. These results suggest the specificity of this marker for lettuce LMV resistance, making easier the screening and incorporation of resistance by marker-assisted selection.

From the tests with 22 SSR markers amplifying large fragments, the reaction with the pair of primers from literature PKCT47F/PKCT47R [18] was shown to be polymorphic for parents of the pumpkin Cabotiá Jabras, allowing the differentiation between progenitors and hybrids. **Figure 5** shows the PCR amplification

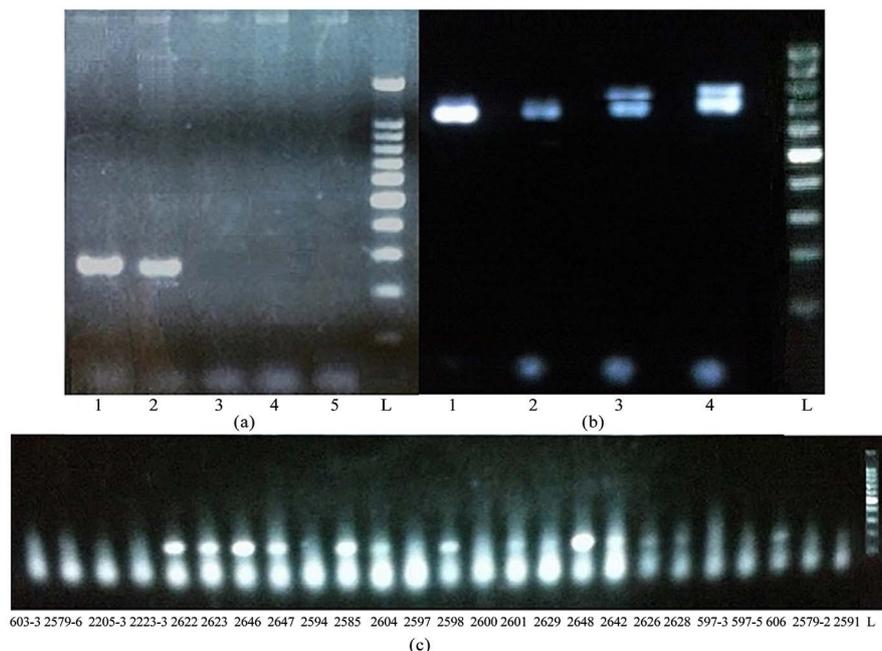


Figure 1. PCR profile of tomato hybrids and varieties. (a) using the 562 JB/828JB primers for *Ty-1* gene of resistance to TYLCV where 1 = 2648, 2 = Aguamiel, 3 = Carina, 4 = Santa Cruz, 5 = Santa Clara, L = 100 bp ladder. (b) using SCAR C19F/SCAR C19R primers for samples numbers 1, 2, 3 and 4. (c) Using the 562 JB/828 JB primers for twenty five tomato accessions of HORTEC SEEDS.

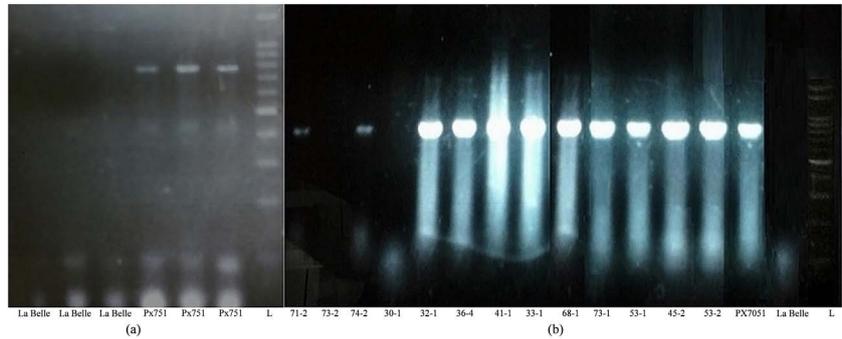


Figure 2. PCR profile using the 36 F/826 R primers for ZYMV resistance of zucchini samples. (a) with three DNA concentrations of the resistant hybrid Px705 and the susceptible cultivar La Belle. (b) thirteen accessions of HORTEC SEEDS, besides the resistant hybrid Px705, the susceptible cultivar La Belle and the 100 bp ladder (L).

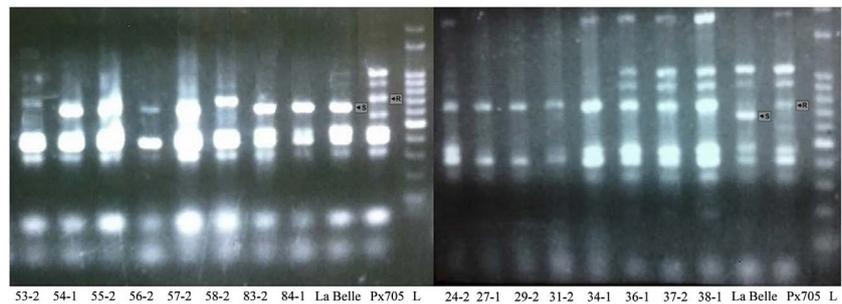


Figure 3. PCR profile for PRSV resistance of zucchini samples using the 408 F/552R primers, for sixteen accessions (HORTEC SEEDS), two resistant hybrids (Px705), two susceptible cultivars (La Belle) and 100 bp ladders (L). Insertion of arrow R for resistance and arrow S for susceptibility.

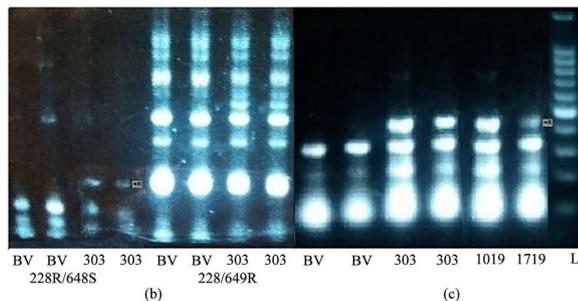
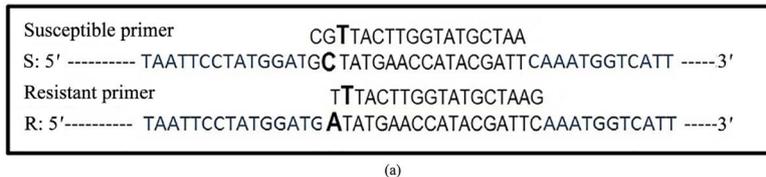


Figure 4. (a) Base pairing of susceptible (S) and resistant (R) lettuce genomes in the developing region of the susceptible and resistant reverse primers; (b) PCR profile of lettuce cultivars Babá de Verão (susceptible) and Brasil 303 (resistant) with two sets of primers for lettuce LMV resistance: 228 R/648 S and 228 R/649 R; (c) PCR profile for lettuce LMV resistance using the 228 R/648 S primers, for the same cultivars at two DNA concentrations, the resistant accessions 1019 and 1719, and 100 bp ladder (L). Insertion of arrow R for resistance.

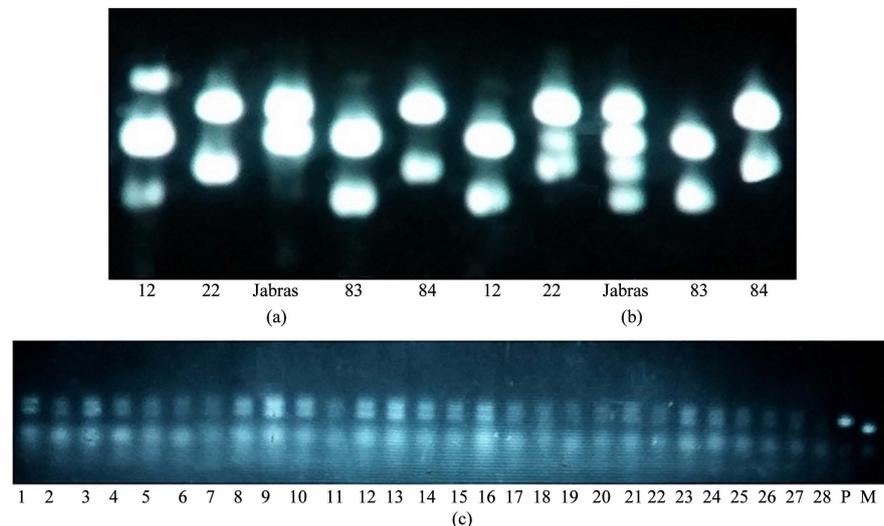


Figure 5. PCR amplification profile of pumpkin samples. (a) using 15 μM of the primers PKCT47F/PKCT47R, of the maternal (1,405,012 = 12 or 145,083 = 83) and paternal (135,122 = 22 or 145,084 = 84) progenitors and the respective Cabotíahybrid known as Jabras. (b) using 10 μM of primers for the same samples. (c) using 10 μM of primers for 28 accessions along with the progenitors, maternal 1,405,012 (M) and paternal 135,122 (P).

profile using the PKCT47F/PKCT47R primers of the hybrids and progenitors (maternal: EEH03-1405012 or 12 and paternal: EEH02-135122 or 22) where, even with different amounts of primers (5A and 5B), the hybrid profile can be recognized. **Figure 5(c)** shows 28 accessions along with progenitors where the first 27 accessions presented the hybrid profile.

All the markers presented allowed the easy visualization of the desirable character with only a single PCR reaction from genomic DNA, making easier the improvement of horticultural plants as tomato, zucchini and lettuce.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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