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PROTOCOLS AND METHODS

Rapid genotyping in tomato by VPCR using agarose gel-resolvable InDel markers

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Abstract

Insertion/deletion (InDel) markers are second most abundant polymerase chain reaction (PCR)-based molecular markers having enormous applications in genotyping and molecular breeding in different crops. Although standard polymerase chain reaction (PCR) for DNA amplification generally takes ~ 1.5 to 2 h, small amplicons can be effectively generated using dynamic heating and cooling through PCR with "V"-shaped thermal profile (VPCR) in ~ 15 to 20 min. Here, we evaluated the applicability of a partly modified VPCR method for amplifying InDels of tomato genome. Out of the 31 InDel markers tested in 15 diverse tomato genotypes, 29 markers resulted in sharp amplicons, where 26 markers were found to be polymorphic. Using this method, the individual DNA amplification reactions could be completed within ~ 30 min. The method was effective for primers varying in melting temperature (T_m) and GC contents. Furthermore, the need for empirically determining suitable annealing temperature could be bypassed using this generalised thermal profile. Through our results, we advocate the use of this method of DNA amplification in other plants to achieve rapid genotyping using standard molecular biology equipments and procedures.

Keywords High throughput genotyping · Insertion/deletion (InDel) markers · Molecular breeding · Rapid DNA amplification

Advancements in the field of molecular markers have revolutionized plant breeding. Among different types of molecular markers, insertion and deletion (InDel) markers, the second most abundant polymorphism in genomes, have massive importance in humans as well as other crop plants' genotyping (Păcurar et al. 2012; Montgomery et al. 2013; Wu et al. 2013; Zeng et al. 2013; Yang et al. 2014). In the important vegetable crop tomato (*Solanum lycopersicum* L.), InDel markers have been explored for diversity analysis (Phan et al. 2016; Jin et al. 2019), genetic association study with fruit quality traits (Liu et al. 2017), genotyping at disease resistance locus (Kim et al. 2017), and many more. The short InDel markers, capable of producing standard agarose gel-resolvable polymorphism are being developed in different crops as appreciable tools for low-cost genotyping (Hu et al. 2020; Kizil et al. 2020; Adedze et al. 2021). Such genotyping requires three basic steps: isolation of genomic DNA, polymerase chain reaction (PCR) for amplification of specific locus and agarose gel electrophoresis for detection of length polymorphism of amplicons. We have found the rapid genomic DNA isolation method (Kumar et al. 2017) and high-voltage agarose gel electrophoresis using sodium borate buffer (Brody and Kern 2004) to significantly cut-short the time requirements for the 2 of the 3 basic steps mentioned before. As the time required for DNA amplification through PCR is the main time-consuming step, a method capable of rapid DNA amplification should permit high-throughput genotyping even with standard lab-equipments.

The general thermal profile of a PCR using standard *Taq* DNA polymerase contains initial denaturation step at ~ 94 °C (for 2–4 min), 25–40 cycling steps of denaturation at ~ 94 °C (for ~ 30 s), annealing at proper temperature (for ~ 30 s) and extension at 72 °C (for ~ 30 s, if the target amplicon size is ~ 500 bp) followed by final extension at 72 °C (for 7–10 min) and hold at an appropriate (4 °C/25 °C)

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temperature. This makes the time required to complete the DNA amplification process to be ~ 1.5–2 h. However, in the recent past, it has been proven that, particularly for the small amplicons, the three processes of the cycling steps in PCR (i.e., denaturation, annealing and extension) can be effectively completed during the alternate heating and cooling procedure (Chen et al. 2019). In this way, rapid amplification is achieved using dynamic heating and cooling using only two temperatures during cycling step. The temperature–time curve, in this way, forms a repeated "V" shape, which gives this rapid DNA amplification method its name, VPCR (Chen et al. 2019, 2021).

As VPCR is a remarkable technique to cut-short the time required for DNA amplification, we tested the applicability of the same in genotyping 15 lines of tomato, including red fruited lines (H-86, BRDT-1, CLN-B, CLN-1621-L, Kashi Chayan, VRTOLCV-16, VRTOLCV-32 and H-88-78-1), pink fruited line (IIHR 2614), yellow fruited lines (VRKB-08, VRKB-09, VRKB-12 and VRCYT-04), orange fruited line (2018/TOINDVAR-05) and a purple fruited line (Purple Tomato, Hazra et al. 2018). Before applying the VPCR technique in tomato genotyping, the method was tested using plasmid DNA as template. The pRS::SlGLPH recombinant plasmid (Kumar et al. 2016) contains the *SlGLPH* (*Solanum lycopersicum* germin like protein H, Chattopadhyay 2014) coding DNA sequence (CDS). A set of 7 gene-specific primers (1 forward and 6 reverse primers, Supplementary Table 1) were tested separately to examine the efficiency of VPCR in generating amplicons of different lengths (ranging from 80 to 230 bp). The 10 µl reaction mix contained 2 µl of plasmid DNA (~ 20 ng) as template, 5 pmol of forward and reverse primers and 2 µl of 2X *Taq* Mix [0.25 U/µl Taq DNA polymerase, 2X PCR buffer, 0.4 mM dNTPs, 3.2 mM $MgCl_2$ and 0.02% (w/v) bromophenol blue; BioLit, SRL]. The VPCR was performed in Veriti 96 well thermal cycler (Applied Biosystems) using 0.2 ml flat cap thin-walled PCR tubes (Tarsons). With minor modifications, the thermal profile for VPCR was set as initial denaturation at 94 °C for 4 min followed by 30 cycles of dynamic heating and cooling (i.e., 94 °C for 0 s and 55 °C for 0 s). The programme ended with a final extension at 72 °C for 2 min and hold at 4 °C for 2 min. To ensure amplification of longer fragments, the 55 °C to 94 °C temperature increment step was performed with 50% ramp rate (resulting in ~ 1.3 °C/s temperature increment in our instrument set up). The resulting amplicons were separated through electrophoresis for 30 min at 120 V in 2.5% (w/v) agarose gel (containing 0.5 µg/ml ethidium bromide) in 5 mM sodium borate buffer (Brody and Kern 2004) and imaged in a gel documentation system (gelLITE, Cleaver Scientific). It was found that all the amplicons with different lengths (i.e., 80 bp, 110 bp, 140 bp, 170 bp, 200 bp and 230 bp) could be efficiently amplified through the VPCR method (Supplementary Fig. Sf1).

In the recent past, a large number of InDel markers have been reported in tomato (Yang et al. 2014). From these markers, we selected 31 markers (Supplementary Table 2), where the length of the insertion/deletion is ≥ 20 bp [so that the generated polymorphism can be detected through standard 2.5% (w/v) agarose gel electrophoresis]. For tomato, genomic DNA isolated from leaf discs (~ 6 mm diameter, collected uniformly through punching the lid of the 1.5 ml micro centrifuge tube on the fully expanded leaves) through a rapid method (Kumar et al. 2017) from the 15 selected tomato genotypes was subjected to VPCR using these InDel markers. Among these 31 tested markers, only 2 markers (sli2645 and sli2282) failed to give amplification through VPCR and 3 markers (sli394, sli536 and sli572) were observed to be monomorphic, as revealed through 2.5% agarose gel electrophoresis (Supplementary Fig. Sf2). To further examine the failed markers, standard 3-step PCR (initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s followed by final extension at 72 °C for 7 min and hold at 4 °C for 2 min) was performed using the genomic DNA isolated from 4 genotypes (i.e., H-86, BRDT-1, CLN B and CLN-1621-L). Interestingly, the marker sli2645 failed to amplify any prominent band even through standard PCR, whereas the marker sli2282 produced amplicon of ~ 1200 bp (Supplementary Fig. Sf3). This indicates a primer-specific issue in case of the marker sli2645, whereas for the marker sli2282, the target amplicon was far beyond the capacity of VPCR, as only smaller amplicons (up to 230 bp, as experimentally validated in this study) could be amplified in the dynamic heating process of VPCR.

Amplification results of the 26 polymorphic and agarose gel-resolvable InDel markers obtained through the partly modified VPCR method (as mentioned earlier) are presented and summarised in Fig. 1 and Table 1, respectively. Among the 26 polymorphic markers, 7 markers (i.e., sli2311, sli528, sli554, sli564, sli565, sli628 and sli653) yielded multiple bands in some samples, whereas rest of the 19 markers produced polymorphic single bands (Fig. 1). Except the marker sli685, all of these 19 polymorphic markers were found to be bi-allelic in nature, whereas 3 distinct polymorphic bands (band size of ~ 139 bp, ~ 151 bp and ~ 163 bp) were amplified by the marker sli685 (Fig. 1). The marker polymorphism data was used to generate Jaccard's similarity index values and the dendrogram (Supplementary Fig. Sf4) using D-UPGMA programme (http://genomes.urv.cat/UPGMA). Lowest Jaccard's similarity index values were obtained between the genotypes BRDT-1 and IIHR-2614, Kashi Chayan and H-88-78-1, VRTOLCV-16 and H-88-78-1, and VRCYT-04 and H-88-78-1. The polymorphic information content (PIC) values of the InDel markers (Table 1) were calculated as described previously (Chattopadhyay

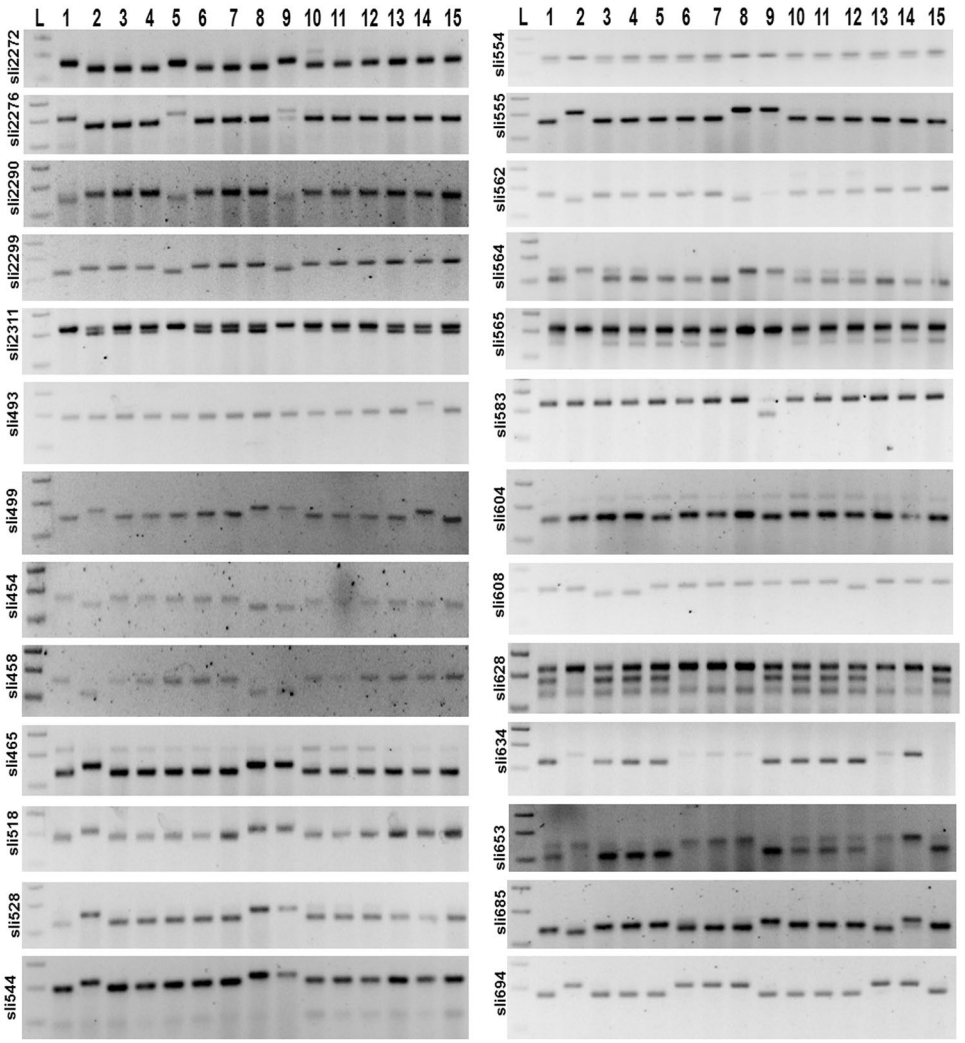

Fig. 1 VPCR-based genotyping of tomato lines. Inverse image of ethidium bromide stained 2.5% (w/v) agarose gels showing polymorphic amplicons generated by 26 InDel markers in 15 tomato lines by partly modified VPCR method. Lane *1* = H-86, *2* = BRDT-1, *3* = CLN B, *4* = CLN-1621-L, *5* = IIHR 2614, *6* = Kashi Chayan, *7* = VRTOLCV-16, *8* = VRTOLCV-32, *9* = H-88–78-1, *10* = VRKB-08, *11* = VRKB-09, *12* = VRKB-12, *13* = VRCYT-04, *14* = 2018/TOINDVAR-05 and *15* = Purple Tomato. *L* = 100 bp DNA ladder (BioLit, SRL)

et al. 2008), which in this case ranged from 0.12 (for sli493 and sli583) to 0.50 (for sli634). Although the molecular diversity revealed by the limited number of InDel markers failed to resolve individual genotypes, some interesting observations could be recorded. For example, the InDel-based genotyping put 3 of the 4 yellow-fruited tomato lines (VRKB-08, VRKB-09 and VRKB-12, except VRCYT-04) in the same cluster (Supplementary Fig. Sf4). All of these three yellow-fruited lines contain the same single nucleotide polymorphism (SNP) in the *phytoene synthase 1* (*Psy1*) gene, whereas yellow fruit colour in VRCYT-04 is probably due to a separate deletion in the *Psy1* gene (data not shown). Moreover, the genotypes BRDT-1, VRTOLCV 16, VRTOLCV 32, VRCYT-04 and Kashi Chayan have been documented to contain the *Ty3* resistance allele (Maurya et al. 2020) that governs resistance against the tomato leaf curl disease. Very interestingly, VRTOLCV 32 and BRDT-1 clustered together and VRCYT-04, VRTOLCV 16 and Kashi Chayan clustered together (Supplementary Fig. Sf4). This result indicated that these InDel markers (along with more number of the InDel markers) are useful tools for molecular phylogenetic studies.

Next, we investigated the applicability of the VPCR technique in determining heterozygosity at particular loci. This is quite pertinent as most of the cultivated tomato lines are hybrids. For this purpose, genomic DNA isolated from the five F_1 plants of the cross-combination CLN B × 2018/TOINDVAR-05 were subjected to VPCR using four parental polymorphic markers (i.e. sli493, sli608, sli634 and sli499). All of the markers could generate both parent-specific amplicons in all the 5 F_1 plants, proving their heterozygosity in all the 4 tested loci (Fig. 2). Thus, suitability of VPCR in determining heterozygosity claims its application in seed industries for rapid purity testing of hybrid seed lot at molecular level.

We further tested the applicability of VPCR for different amounts of tomato genomic DNA (used as template) isolated in different methods. For this purpose, we used three different methods: (a) the standard organic extraction method, (b) the rapid method (Kumar et al. 2017) and (c) the alkali

Table 1 Amplification results of the 26 polymorphic and agarose gel-resolvable InDel markers in 15 tomato genotypes

S.N.	Marker	Expected amplicon* (bp)	Observed amplicons (bp)	PIC value
1	sli2272	139	139/161	0.32
2	sli2276	165	165/203	0.32
3	sli2290	159	139/159	0.32
4	sli2299	141	121/141	0.32
5	sli2311	198	198/222	0.16
6	sli493	193	193/221	0.12
7	sli499	145	145/168	0.39
8	sli454	172	146/172	0.32
9	sli458	158	111/158	0.32
10	sli465	136	136/158	0.32
11	sli518	192	192/218	0.32
12	sli528	121	121/150	0.40
13	sli544	193	193/213	0.32
14	sli554	150	150/170	0.16
15	sli555	163	163/205	0.32
16	sli562	174	154/174	0.23
17	sli564	107	107/139	0.40
18	sli565	150	150/213	0.16
19	sli583	162	162/228	0.12
20	sli604	151	151/141	0.23
21	sli608	174	174/200	0.32
22	sli628	174	134/174/221	0.16
23	sli634	122	122/150	0.50
24	sli653	107	107/145	0.46
25	sli685	139	139/151/163	0.39
26	sli694	177	177/204	0.48

bp base pairs; *PIC* polymorphic information content; highest and lowest values are indicated by underline

*Expected amplicon lengths were deduced through in silico PCR using the primer sequences (Supplementary Table 2) in SolGenomics database (https://solgenomics.net/)

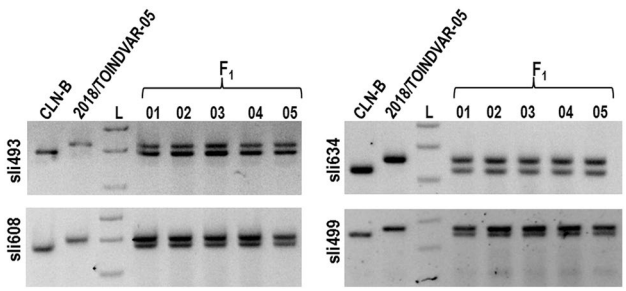

Fig. 2 Applicability of VPCR in determining heterozygosity. Inverse image of ethidium bromide stained 2.5% (w/v) agarose gels showing amplicons generated by 4 parental polymorphic InDel markers (sli493, sli608, sli634 and sli499) in tomato genotypes CLN B and 2018/TOINDVAR-05 and the derived 5 F_1 hybrid plants. $L = 100$ bp DNA ladder (BioLit, SRL)

lysis method. As we target to achieve rapid genotyping, all the methods bypass the time-consuming step of quantitative and/or qualitative assessment of isolated genomic DNA and focus on processing equal amount of leaf tissue (~6 mm diameter, collected uniformly through punching the lid of the 1.5 ml micro centrifuge tube on the fully expanded leaves). In case of the standard method, the leaf tissue was crushed in 400 µl of isolation buffer [100 mM Tris–HCl (pH 8.0), 50 mM EDTA (pH 8.0), 500 mM NaCl and 1% (w/v) SDS with 0.2% (v/v) β-mercaptoethanol added just before use]. Then 128 µl of 5 M CH_3COOK was added and the tubes were vortexed briefly. Following centrifugation at 10,000 RPM for 1 min, the supernatant was collected in a fresh tube and 400 µl of phenol:chloroform:isoamyl alcohol [25:24:1 (v/v)] was added. Following shaking and centrifugation at 10,000 RPM for 3 min, the upper layer was collected in a fresh tube and 400 µl of chloroform:isoamyl alcohol [24:1 (v/v)] was added. Following shaking and centrifugation at 10,000 RPM for 3 min, the upper layer was collected and DNA was precipitated using 400 µl of isopropanol. The resulting pellet was washed in 70% (v/v) ethanol, dried and dissolved in 200 µl of molecular biology grade water. In case of the rapid method, the supernatant obtained after the first centrifugation step was collected in a fresh tube and DNA was precipitated using 400 µl of isopropanol. Identical steps of the standard method were performed for drying the DNA pellets and dissolving the same. For the DNA samples obtained through standard and rapid method, crude, twofold, fivefold and tenfold dilutions were prepared for use in VPCR. For the alkali lysis method, the leaf tissue was crushed in 200 µl of 0.25 N NaOH and the samples were centrifuged at 10,000 RPM for 1 min. The supernatant was collected in a fresh tube and fivefold, tenfold, 20-fold and 30-fold dilutions were prepared in dilution buffer (10 mM Tis-HCl, 0.1 mM EDTA, pH 8.0). For all the methods and dilutions, 2 µl of template was used in VPCR. Using the H-86 genotype and the sli555 marker, we observed the VPCR method to be equally effective for all the methods and all the DNA dilutions (Fig. 3A). For comparison, standard 3-step PCR (initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s followed by final extension at 72 °C for 7 min and hold at 4 °C for 2 min) was performed with the same set of samples and the sli555 marker, where identical result was obtained (Fig. 3B). This result established VPCR as an undeniable replacement of the time consuming standard 3-step PCR for amplifying small fragments. Moreover, suitability of different DNA dilutions/methods in generating sufficient VPCR amplicons should provide confidence to the user in bypassing qualitative/quantitative assessment of DNA, particularly when rapid genotyping is the target.

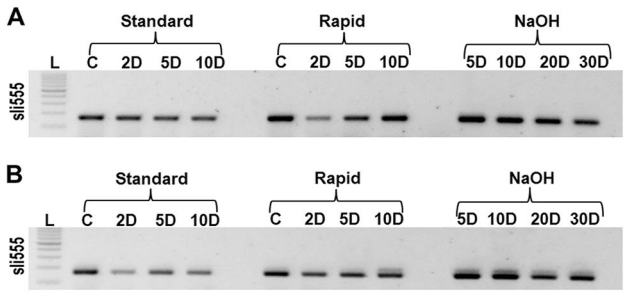

Fig. 3 Suitability of VPCR in utilizing DNA template prepared in different methods. A. Inverse image of ethidium bromide stained 2.5% (w/v) agarose gels showing amplicons generated by the InDel marker sli555 through VPCR using different amounts of template DNA prepared from tomato genotype H-86 using standard, rapid and alkali lysis (NaOH) methods. B. Inverse image of ethidium bromide stained 2.5% (w/v) agarose gels showing amplicons generated by the InDel marker sli555 through standard 3-step PCR using different amounts of template DNA prepared from tomato genotype H-86 using standard, rapid and alkali lysis (NaOH) methods. *C* = crude sample; *2D* = twofold diluted sample; *5D* = fivefold diluted sample; *10D* = tenfold diluted sample; *20D* = 20-fold diluted sample; *30D* = 30-fold diluted sample. *L* = 100 bp DNA ladder (BioLit, SRL)

Next, we investigated whether the time requirement of VPCR could be further reduced. For this purpose, crude, twofold, fivefold and tenfold diluted DNA sample of H-86 genotype, isolated in rapid method was subjected to 25 cycles/30 cycles/35 cycles VPCR using the sli555 marker. While 25-cycle VPCR failed to generate detectable amplicons in all the dilutions, amplicons could be generated in crude and twofold dilution samples even after 30 cycles (Supplementary Fig. Sf5.A). This indicates a comparable sensitivity of VPCR and standard 3-step PCR. Another way of reducing the time requirement of VPCR was to increase the ramp rate of the 55 °C to 94 °C temperature increment step. However, when 75% or 100% ramp rate for the aforementioned step was applied, VPCR failed to generate detectable amplicon in all 4 DNA dilutions tested (Supplementary Fig. Sf5.B). This result emphasised the importance of reduced ramp rate for the 55 to 94 °C temperature increment step of VPCR. The reduced ramp rate provides sufficient time to *Taq* DNA polymerase for primer extension during dynamic heating and cooling.

The application of VPCR (with minor modifications, as mentioned earlier) in rapid amplification of tomato InDel markers reduced the time required for PCR significantly, where individual reactions were completed in ~ 30 min. The gene-specific primers used for amplification from plasmid DNA had the melting temperature (T_m) ranging from 52.80 to 69.54 °C and GC content ranging from 31.82 to 64% (Supplementary Table 1). Interestingly, the generalised thermal profile of VPCR used in this study could efficiently use all these primers to generate specific sharp amplicons of different length (Supplementary Fig. Sf1), which advocates the suitability of this generalised VPCR profile with the primers having variation in T_m and GC content. In corroboration, the used tomato InDel markers (Table 1) varying in T_m values (ranging from 53.20 to 61.40 °C) and GC content (ranging from 41.67 to 60%) were successfully used in this study (Fig. 1). Thus this generalised VPCR technique could eliminate the cumbersome steps of empirical optimization of annealing temperature for individual primer pairs and could provide more options (in terms of T_m values and GC content in the primers) in designing specific primers for reliable PCR. The suitability of VPCR in detecting heterozygosity (Fig. 2) should find potential application in seed industries for rapid testing of purity in hybrid seed lot. Furthermore, we tested whether VPCR-based genotyping could be applied in other crops. For this purpose, four genotypes of brinjal and rice were subjected to VPCR using two simple sequence repeat (SSR) markers of brinjal and one SSR marker of rice. In both the cases, we successfully obtained sharp amplicons (Supplementary Fig. Sf6). Hence, on the basis of our results, we advocate the use of VPCR as a potent rapid genotyping method in different crops.

In conclusion, we validated the utility of VPCR for rapid genotyping using tomato as experimental material. The alkali lysis method was the fastest to provide template (up to 30 fold dilution) suitable for VPCR. In a probable situation, where 24 marker-genotyping has to be performed with 24 genotypes, alkali lysis method followed by VPCR can be a real smart choice, where the entire genotyping task can be completed in a single 8 h-working day [i.e., 1 h for DNA isolation and VPCR set preparation, ~ 3.5 h for 6 VPCR in 96-well thermal cycler (4 markers × 24 samples in each run) and ~ 3.5 h for 6 times gel electrophoresis (using 96 lane gels). Apart from research, another very important application of VPCR should be in academics. The alkali lysis method of DNA isolation from a few genotypes followed by VPCR with a couple of markers and gel electrophoresis of the amplified products can be happily completed within 2 h. Thus, it allows the teacher/instructor and the students to complete a comprehensive practical class of plant biotechnology within 2–3 h of stipulated time. Finally, VPCR allows rapid genotyping using standard lab-equipments and saves the invaluable research input, i.e. time.

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Author contributions TC conceived the idea. SS and TC performed the experiments. SA, TC and SS analysed the results. All the authors took part in preparation and correction of the manuscript. All the authors read the final manuscript and approved it.

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Data availability All data generated in this research is incorporated in the manuscript and associated supplementary information.

Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Research involving human participants and/or animals No human participants and/or animals were involved in this study.

Informed consent Not applicable as no human participants and/or animals were involved in this study.

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