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Study on Genetic Diversity of Terminal Fragment Sequence of Isolated Persian Tobacco Mosaic Virus

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Abstract

Tobacco mosaic virus (TMV) is one of the devastating plant viruses in the world that infects more than 200 plant species. Movement protein plays a supportive role in the movement of other plant viruses, and viral coat protein is highly expressed in infected plants and affects replication and movements of TMV. In order to investigate genetic variation in the terminal fragment sequence in Iranian TMV isolate, viral RNA was extracted from the infected tobacco leaves and cDNA was constructed using a specific antisense primer, *PSh*60-R1. The coding sequence of movement protein and coat protein was amplified and bi-directionally sequenced using *PSH63*-F/R primers. The results from sequencing were analyzed by Vector NTi software, and the nucleotide sequence was aligned with other TMV isolates of Iran using MEGA 5 and CLC Main Workbenches. Based on the results, the comparison between sequences of movement protein from TMV-ir and movement protein sequence of TMV-U1 reveals six mutations. Also, nucleotide comparison reveals that there are two mutations between coat protein sequence of TMV-ir and TMV-U1. Based on sequencing results, there is the minimum nucleotide distance between TMV-ir and TMV-U1 and maximum distance between isolates of Iran (Br50, TO79, TO32, TA, PU1, TM, G42, TV). Based on the results, we report a new isolate of TMV from Iran that genetically distinct with other Iranian isolates. These results provide good knowledge that could be valuable to designing detection kits and plant breeding programs.

Key words: Coat protein; Movement protein; Mutation; TMV

Introduction

Tobacco mosaic virus (TMV) is a member of Tobamovirus genus and it is an extensively applied model to study the interaction between plant and virus. This virus has a worldwide distribution and infects over 200 plant species including *Solanaceae* family (Choi *et al*., 2009). TMV causes severe losses in the important crops including tomato and pepper (Dawson *et al*., 1986). The full sequence of its genome consists of 6395 nucleotides. It is a single-strand positive-sense ssRNA and encodes four open reading frames (ORF) (Dawson *et al*., 1986). The replicase proteins are translated from the genomic RNA whereas movement protein (MP) and coat protein (CP) are expressed from subgenomic RNAs (Scholthof, 2004). The third ORF encodes the 29-kDa MP and consists of 4903-5709 nucleotides. The fourth ORF encodes the 17.5-kDa CP and consists of 5712-6191 nucleotides (Körbelin *et al.*, *2012*). The Tobamovirus genus consists of 22 definitive and one tentative species (Fauquet *et al*., 2005; Gibbs, 1977; Gibbs *et al*., 2004) which are divided into three subgroups based on natural

host range (Adkins *et al*., 2003; Fauquet *et al*., 2005; Gibbs, 1986; Lartey *et al*., 1996). The full length nucleotides of some members of the Tobamovirus genus have been reported and have been submitted in GenBank database (Goelet *et al*., 1982; Lartey *et al*., 1996). The most important identified isolates of the virus are the U1 (USA) and OM (Japan) (Nozu and Okada, 1968; Siegel and Wildman, 1954; Watanabe *et al*., 1999). Different strains of the virus can cause very devastating symptoms and lead to different diseases in the crops. It is important to identify the properties of virus strains in the plant breeding for special resistance to different strains of viruses (Rast, 1972). Analysis of TMV genome structure and its properties is an important aspect that must be investigated (Choi *et al*., 2009). For example, MP has been used to study the mechanisms of plasmodesmata gating and analysis of gene function. Also, it has been showed that MP can play a supportive role in the movement of other viruses. TMV has been used to investigate how virus movement proteins can cause physical changes in plasmodesmata transit large macromolecules (Scholthof, 2004). There have been several studies about the role of CP and it has been showed that the CP has an important role in the virulence and evolution in different strains (Saito *et al*., 1987). It is reported that after recombination between two species, CP could cause a necrotic reaction in the plant (Saitou and Nei, 1987). In the other study, recombination between the wild type TMV and nitrous acid mutant showed that mutation in CP nucleotide 6157 which could replace serine instead of phenylalanine in amino acid 148, resulting in necrotic local lesion (Knorr and Dawson, 1988). It is also reported that CP is important in symptomology (Dawson *et al*., 1986). Furthermore CP is involved in the movement of virus and its systemic extension (Hull, 2001). There are several studies about variation in CP to investigate the relationship between different isolates of TMV (Choi *et al*., 2009; Ghavidel *et al*., 2014; Körbelin *et al*., 2012). In the present study, we investigated the determination of MP and CP gene sequences in Persian TMV isolate (TMV-ir) and also compared our results with different isolates in GenBank.

Materials and Methods

Sample preparation of TMV

Sample of infected tobacco leaves (*Nicotiana tabacum* L.var. Turkish) were supplied in (liquid nitrogen from plant pathology department of Ferdowsi University of Mashhad.

Virus RNA extraction

Total RNA extraction was isolated from symptomatic tobacco leaves by column RNA isolation kit-π (DENA zist, Mashhad, Iran) according to the instruction of the kit.

Primer design

The complete nucleotide sequence of TMV was searched from NCBI GenBank and TMV sequence isolate TMV-U1 was recovered with the NC-001367 accession number. ORF position of MP and CP was determined by Vector NTi software. Forward primer PSh63-F (5'-CAT AAG ACC GCC CCT CCA G-3) and reverse primer PSh60-R (5'-CGT TAT CGT ACG CAC CAC GTG-3') were designed by Primer Premier Ver.6 software for detecting and amplifying the MP and CP genes of TMV. primers were designed around 100 base pairs upstream and 100 base pairs downstream the terminal fragment sequence containing MP and CP genes respectively for annealing the primers on the MP and CP gene.

cDNA Synthesis

cDNA synthesis was carried out according to column RNA isolation kit-π (DENAzist, Mashhad, Iran) in a reaction containing 200 ng of total RNA, 50 pmol reverse primer PSh60-R and 3µl DEPC water (Diethylpyrocarbonate, DENAzist, Iran). The reaction was incubated at 70˚C for 5 minutes, then after chilling on ice, 4 µl of 5x reaction buffer, 2 µl dNTP 10 mM and 3 µl DEPC treated water were added. Reaction was incubated at 37˚C for 5 minutes. Finally, 1 U Prime MMLV Reverse transcriptase (GENET BIO, Korea) was added and the reaction was incubated at 37˚C for 60 minutes. The reaction

was stopped by heating at 70˚C for 10 minutes and chilled on ice.

Amplifying terminal fragment sequence containing MP and CP genes

Amplification of the fragment was performed with PCR method using *PSh63*-F as the forward primer and *PSh60*-R as the reverse primer in a 20 µl reaction containing 1µl cDNA; 0.6 µl of 10 mM dNTPs; 2 µl of 25 mM $MgCl_2$, 2 µl of 10x, PCR buffer, 5 pmol of primers and 1 U *Taq* DNA polymerase. Amplification was carried out according to the Table 1 in a thermocycler (MWG-BIOTECH Primus25, Germany) using 1 cycle initial denaturation at 93˚C for 300 sec., denaturation followed by 35 cycles at 92˚C for 45 sec., annealing at 60˚C for 60 sec., elongation at 72˚C for 60 sec., and a final extension at 72˚C for 300 sec. PCR product was checked on 1% agarose gel at 1 hour and was colored by Green viewer and was imaged by gel document (Uvitec GA59000, UK).

Purification and sequencing of amplified fragment

The amplicon of TMV (MP and CP gene) was obtained from the agarose gel and was purified using gel extraction kit (QIAGEN, US) and the results were sequenced bi-directionally using PSh63-F and PSh60-R primers by MACROGEN (Seoul, South Korea). The sequencing results were analyzed using Vector NTi and DNA baser software. The results were submitted to GenBank.

Phylogenetic analysis

The obtained sequences were compared with available TMV sequences in the GenBank database (Table 1) and multiple sequence alignments were performed by MEGA, Ver. 5.2 software (Tamura *et al*., 2011). Also, the translations of nucleotide sequences of MP and CP to amino acid sequences were performed by MEGA, Ver. 5.2 software. The amino acid sequences of MP and CP TMV-ir were compared with other isolates from Iran using CLC Main Workbench, ver. 5. Evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The tree was drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and were in the units of the number of amino acid substitutions per site. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011).

Table 1. Properties of TMV isolates which were used in phylogenetic analysis

Strain or isolate	Accession no	origin	Host
TMV-U1	NC001367	Germany	*Tobacco*
TMV-Ohio V	FR878069	USA	*Petunia*
Br50	KF527474	Iran (Savejbolagh)	*Brassica oleracea*var.capitata
TO79	KF527472	Iran (Tehran)	*Capsicum frutescens*var.longum
TO32	KF527470	Iran (Varamin)	*Solanumlycopersicum*
TA	HQ593617	Iran (Alborz)	*Tomato*
PU1	HQ593618	Iran (Shiraz)	*Pumpkin*
TM	HQ593619	Iran (Eastazarbaijan)	*Tomato*
G42	HQ593620	Iran (Golestan)	*Eggplant*
TV	HQ593621	Iran (Tehran)	*Tomato*

Results and Discussion

The results of RNA extraction and cDNA construction were confirmed by PCR and specific primers. Specific primers aiming at 3′ end of the genome were used and the fragment of the expected size was 1442 bp (Fig. 1A). Results of electrophoresis of PCR product, confirmed a single DNA fragment of expected size (about 1500 bp) (Fig. 1B). The fragment was sequenced bi-directional using specific primers. The deduced nucleotide sequences were 1324 bp. The fragment was contained the MP (799 nt), CP (480 bp), 25 nt upstream the MP

and 11 nt upstream and downstream the CP respectively. The beginning of the fragment was at the nucleotide position 4878 and the end of it was at nucleotide position 6202 and it was containing MP and CP sequences. Sequence of the fragment was submitted to GenBank.

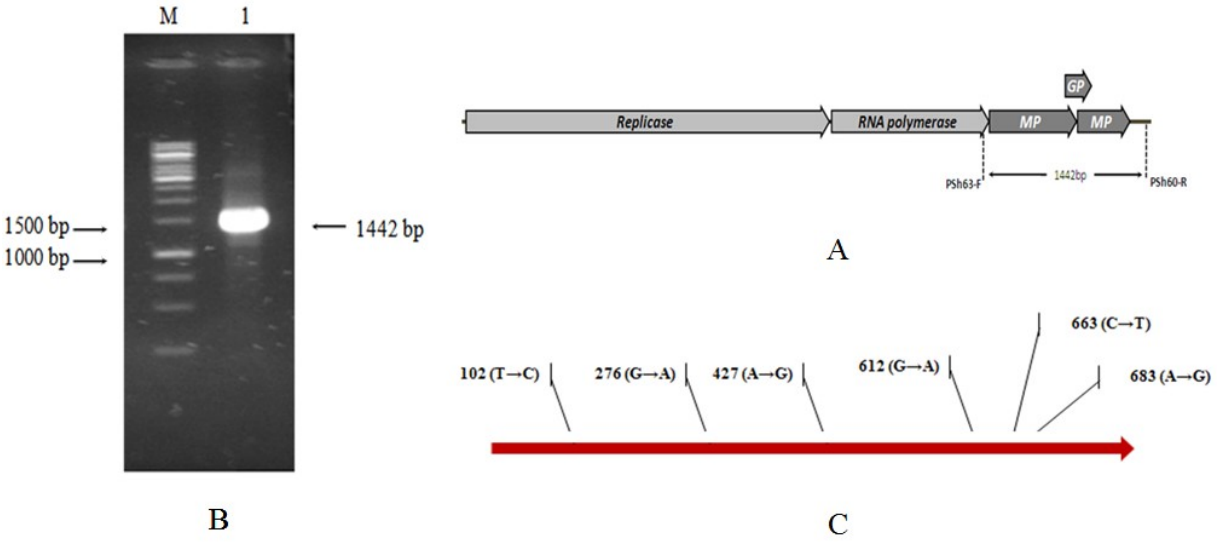

Fig. 1. Genome organization of tobacco mosaic virus and electrophoresis analysis of PCR product using specific primers: (A) Coding proteins and position of the placement specific primers *PSh-63F/60R* and the size of the amplified fragment: (B) Specific band amplifying by product of cDNA made of virus RNA; M. 1Kb DNA Ladder. Comparing the mutation and their position at coding movement protein between tobacco mosaic virus isolate of Iran (TMV-ir) and reference sequence (TMV-U1) (C)

Movement protein

Sequence of TMV-U1was selected as a reference to compare sequence of MP TMV-ir whit other isolates of TMV in NCBI GenBank and nucleotide sequence of MP at TMV-ir was aligned with TMV-U1 using MEGA5. Based on the results, TMV-ir with 799 bp was different from TMV-U1 only in six nucleotide positions (Fig. 1).

These results revealed the proximity relationship between TMV-ir and TMV-U1. In previous reports of TMV was showed the overlap protein coding sequences were showed in different isolates of TMV. It is reported that MP of TMV-Ohio V revealed 84% identity of nt with TMV-U1 (Körbelin *et al.*, 2012). Mutations in nucleotide sequences could be synonym or non-synonym in protein level. Therefore, MP sequence of the isolates was compared at protein level. Comparing and analysis of MP amino acids in two isolates was performed by MEGA5. Analysis of the sequences in TMV-ir and TMV-U1 were similar showed that, only two mutations among 266 amino acids were present at protein level. Based on the results of amino acid sequence of TMV-ir, Isoleucine was changed to the Valine and Asparagine was changed to Serine at position 143 and 228, respectively. In the phylogenetic tree (Fig. 3) these isolates grouped in the same group which confirmed high similarity between them. The effects of mutation in functional region of MP have been reported (Chen *et al*., 2000; Kahn *et al*., 1998). For example, deletion of some amino acids influences MP-PME (pectin methyl esterase) binding and blocks the MP to mediate the spread of viral infection (Chen *et al*., 2000).

Furthermore, amino acid sequence of MP was compared with three isolates of Iran to investigate similarities and differences between new isolate with other reports of TMV in Iran. According to Table 1, isolates are from diverse hosts and was collected from various places.

Alignment and comparing of MP amino acid sequence deduced from translation of TMV-ir

with other isolates of Iran was revealed a few conserved regions by CLC Main Workbench software, whereas conserve region at amino acids 1 to 20 is observable (Fig. 2).

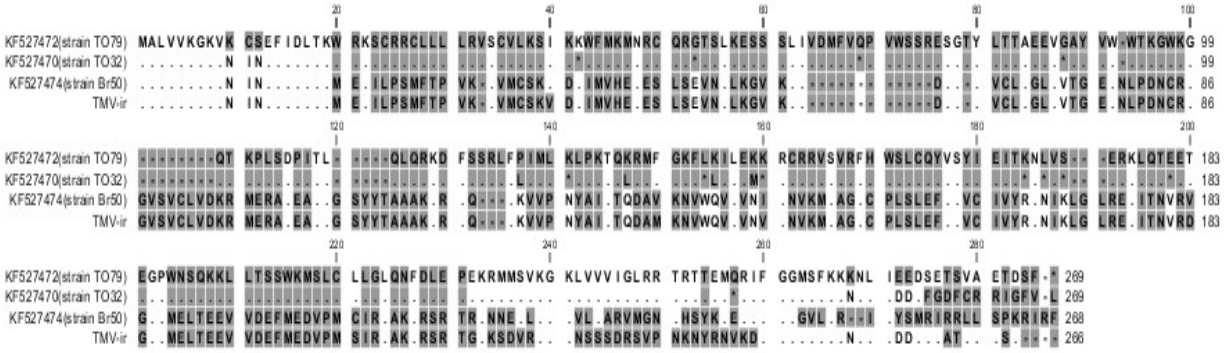

Fig. 2. Multiple alignment of MP amino acid sequence in TMV-ir with the homologous gene of TMV isolates from Savejbolagh (Br50), Tehran (TO79), Varamin (TO32). The dots represent identity amino acids.

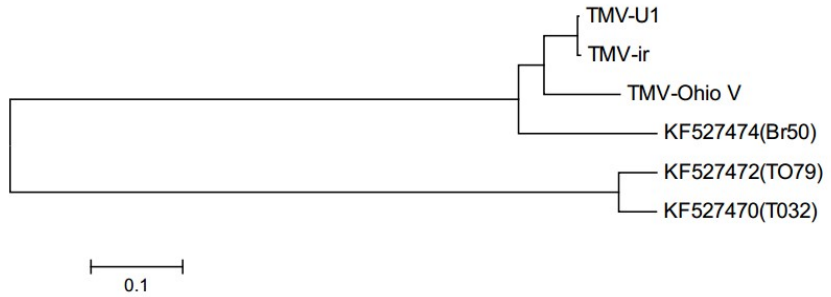

Fig. 3. Phylogenetic tree of TMV-ir with some TMV isolates based on amino acid sequence of MP gene using the Neighbor-Joining method: Information details for each TMV isolate in the phylogenetic tree were shown in table 2.

The relationship between TMV isolates with TMV-ir based on amino acid sequence of MP was shown in Fig. 3. Branch lengths show the evolutionary distances. Analysis based on amino acid sequence clustered isolates in 2 clades. In the phylogenetic tree clustered TMV-ir with reference sequence from Germany (TMV-U1) in a separate group and TMV-Ohio V with Br50 (Savejbolagh, Iran) clustered in other group. Similarity at nucleotide sequences confirmed this result. Two other isolates from Iran (TO79, TO32) clustered in a separate group.

Coat protein

The CP coding sequence of TMV-ir aligned with homologous gene of TMV-U1 was compared with reference sequence TMV-U1 using MEGA 5 software. The results showed two mutations in TMV-ir (480 bp). It is reported that, there was a visible overlap between different isolates of TMV in CP gene and identity of 90% of nucleotides in TMV-U1 and TMV-Ohio V (Körbelin *et al.*, 2012). In other study, CP gene of TMV-U1 was compared with 11 different isolates of TMV by alignment analysis and several mutations at nucleotides were reported, where Some of those mutations changed amino acids (Ghavidel *et al*., 2014).

Comparing of CP amino acid sequence in TMV-ir with 5 isolates, identified similarities and differences between them (Fig. 4). Isolates of Iran have been selected from Alborz (TA), Tehran (TV), Shiraz (PU1), East Azerbaijan (TM) and Golestan (G42) (Alishiri *et al*., 2013). According to Table 1, these isolates have been selected from different hosts. Comparison of CP sequence alignment between TMV-ir and the other isolates from Iran, showed two mutations at TMV-ir amino acid sequence using CLC Main Workbench software. Consequently, glycine was changed to the serine at position

four and serine was changed to the leucine at position 56. Stop codon in six isolates was UGA. Also multiple alignment for CP sequences demonstrated high conservation in CP. These results suggest multifunctional role of CP during plant infection by TMV (Dawson *et al*., 1986) (Fig. 4).

The phylogenetic tree based on CP amino acid sequence is demonstrated in Fig. 5. Branch lengths show evolutionary distances between sequences. The phylogenetic analysis clustered isolates in three groups. In the phylogenetic tree TMV-ir with reference sequence (TMV-U1) clustered in the same group and other isolates from Iran clustered in separate group. Similarity in the nucleotide sequence confirmed this result.

The alignment data was changed to the evolutionary distance matrix that showed the relationship between sequences for the purpose of drawing phylogenetic tree. Accordingly, evolutionary distances based on nucleotide distances between pair of sequences were calculated. The results demonstrated that CP gene nucleotide sequence (480 bp) at Iran TMV isolates, differ at 25 nucleotide positions with TMV-ir, and evolutionary distance between them was 0.052. The minimum evolutionary distance in TMV-ir was observed with reference sequence (TMV-U1). Regardless of numerous reports of TMV from other countries, there are few available reports about molecular organization and phylogenetic analysis of CP gene in Iranian isolates (Alishiri *et al*., 2013). The results of CP phylogenetic analysis showed a low genetic diversity between isolates in one group, whereas indicated that TMV-ir clustered with TMV-U1 in one group and not clustered with Iranian isolates and this result was unexpected. It is reported that a low genetic diversity between CP gene nucleotide sequence in Iranian isolates and all of Iranian isolates clustered in separate group, with 100% identity, had 94% similarity with TMV-U1 (Alishiri *et al*., 2013). Those results suggested the negative selection acting on CP gene.

It is reported that isolates of TMV might be changed by different temperatures (Jones and Dawson, 1978) or in various plant hosts (Yarwood, 1979). It is reported that TMV isolates from different plant species have been under the selection pressures and had an effective role on CP gene diversity (Aldaoud *et al*., 1989). However, these results differed from obtained results by phylogenetic analysis of Iranian isolates that showed low diversity in nucleotide sequence of CP and suggested that the negative selection has acted on CP gene (Alishiri *et al*., 2013). According to the analysis and the length of sequenced fragment containing MP and CP genes (1325 bp) and based on size of MP (799 bp) and size of CP (480 bp), it was resulted that there was no mutation on downstream of CP gene. The 3′-untranslated region (UTR) and poly (A) tail of TMV genomic RNA increased mRNA stability and regulated the efficiency of translation in TMV and had an important role in regulation (Gallie *et al*., 1993). Based on the results, some mutations were shown in this region.

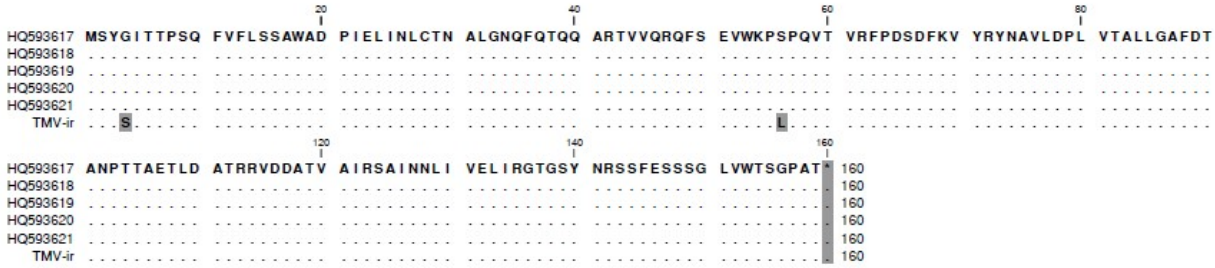

Fig. 4. Multiple alignment of CP amino acid sequence in TMV-ir with the homologous gene of TMV isolates from Alborz (HQ593617), Shiraz (HQ593618), East Azerbaijan (HQ593619), Golestan (HQ593620) and Tehran (HQ593621). The dots represent identity amino acids.

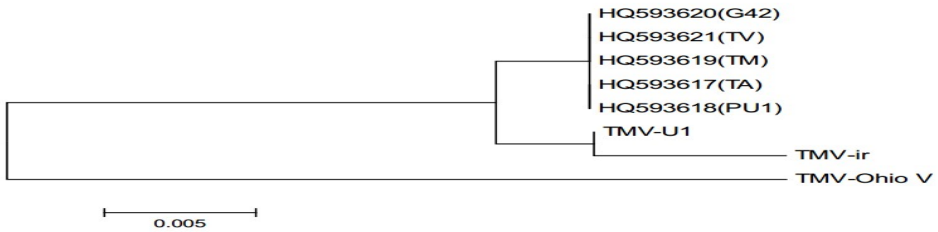

Fig. 5. Phylogenetic tree of TMV-ir with some TMV isolates based on amino acid sequence of CP gene using the Neighbor-Joining.Method: Information details for each TMV isolate in the phylogenetic tree was shown in table 1.

Conclusion

In this study, comparing sequenced MP and CP of one isolate from Iran with TMV Iranian isolates in GenBank to determined correlation between Iranian isolates and geographical distribution of TMV. Also, a few TMV isolates were compared with TMV-ir. All submitted MP and CP genes from Iran in NCBI, have been deployed in this research. Our results showed that TMV-ir was not clustered with Iranian isolates in the same phylogenetic group and TMV-ir clustered with reference sequence (TMV-U1) in one group. Similarity between 2 isolates (TMV-ir, Iran and TMV-U1, Germany) may indicate the existence of common ancestor. However, one explanation is conceivable and that is an adaptation to a common special host and changing in viral fitness by codon changes. Despite differences between TMV-ir with Iranian isolates, we can deduce that no clear correlation between genetic distance and geographical distribution of TMV. In conclusion, these results indicate existence new isolates of TMV in Iran that distinct with other Iranian isolates and knowledge about genomic organization might provide useful tool to designing distinction kits and plant breeding.

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