Research Article

DETERMINATION of GENETIC STABILITY of TOMATO (*Lycopersicon esculentum* Mill.) GRAFTED on TOBACCO (*Nicotiana tabacum* L.) by RANDOM AMPLIFIED POLYMORPHIC DNA ANALYSIS

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ABSTRACT

Grafting has been widely used in agriculture, forestry and horticulture. Grafted plants may exhibit some phenotypic variations from scions and rootstock plants. Existance and possible mechanisms of graft induced genetic variation and inheritance of graft induced characters has been debated among researchers. Use of random amplified polymorphic DNA (RAPD) as genetic marker assay enables detection of genotypic alterations. The prupose of the present study was assessment of genetic stability or any graft-induced changes at molecular level in tomato grafted on tobacco rootstock by RAPD analysis. *Nicotiana tabacum* L. cv. Samsun was used as rootstock and a *Lycopersicon esculentum* Mill. commercial cv. H-2274 was used as scion in grafts. Plants were grafted by cleft grafting method and transferred to soil field in greenhouse. DNA was isolated from fresh leaves of grafted and non-grafted plants 60 days after grafting. RAPD analysis revealed that 8 primers gave a total of 102 bands in the profiles of non-grafted tomato plants. Polymorphism of the grafted tomato plants was caluculated as 2.94% and in concordance, Genomic template stability (GTS) was 97.06%. Succesive grafting with high genomic stability, may improve crop yield in agricultural practices without involvement of genetic transformation.

Key words: Tomato, Tobacco, Scion, Rootstock, GTS

Nicotiana tabacum L. ÜZERİNE AŞILANMIŞ *Lycopersicon esculentum* Mill.'in RASTGELE ÇOĞALTILMIŞ POLİMORFİK DNA ANALİZİ İLE GENETİK STABİLİTENİN BELİRLENMESİ

ÖZET

Aşılama, tarım ve ormancılıkta sıkça kullanılan bir yöntemdir. Aşılı bitkiler, kalem ve anaç bitkilerinden farklı fenotipik özellikler gösterebilir. Yapılan çalışmalar, aşılamanın genetik varyasyonu ve kalıtımı etkileyebileceğini belirtmiştir. Genetik belirteç yöntemi olarak Rastgele Çoğaltılmış Polimorfik DNA (Random Amplified Polymorphic DNA Analysis*;* RAPD) analizinin kullanılması ile genotipik değişimler belirlenebilmektedir. Bu çalışmada amaç, tütün üzerine aşılanmış domates bitkisindeki genetik stabilitenin veya moleküler düzeyde aşı etkili değişimlerin RAPD yöntemi ile tanımlanmasıdır. Aşılama deneylerinde kalem olarak domates bitkisinin (*Lycopersicum esculantum* Mill.) ticari H2274 çeşidi, anaç olarak ise olan tütün bitkisinin (*Nicotiana tabacum* L.) Samsun çeşidi kullanılmıştır. Aşılamalar yarma aşılama tekniği ile yapılmış ve sera ortamına aktarılmıştır. Aşılamadan 60 gün sonra aşılı ve aşısız bitkilerin taze yapraklarından DNA izole edilmiştir. Kontrol olarak kullanılan aşısız domates bitkilerinden 8 adet primer ile elde edilen RAPD profillerde toplam 102 adet bant tespit edilmiştir. Aşılı örneklerdeki polimorfizm oranı %2.94 ve genomik kalıp stabilite oranı ise %97.06 olarak hesaplanmıştır. Yüksek genetik stabiliteye sahip aşılama teknikleri, genetik transformasyon olmaksızın ürün eldesini arttırarak tarımsal uygulamaları geliştirebilmektedir.

Anahtar sözcükler: Domates, Tütün, Kalem, anaç, GKS

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INTRODUCTION

In plant grafting, upper part (scion) of one plant grows on the root system (rootstock) of another plant of the same species or other species of the same genus or family. Grafting has been widely used in agriculture, forestry and horticulture. It is applied to change varieties or cultivars, optimize cross-pollination and pollination, take advantage of particular rootstocks, benefit from interstocks, perpetuate clones, produce certain plant forms, and repair damaged plants. In addition, in agriculture it may increase the growth rate of seedlings for the improvement of crop and vegetable yield, quality, and abiotic-biotic stress tolerance of scion. In agricultural practices grafting has been considered as a method of asexual plant propagation rather than plant breeding.

Grafted plants may exhibit some phenotypic variations, such as stress tolerance and crop yield, from scion and rootstock plants. Existance and possible mechanisms of graft induced genetic variation and inheritance of graft induced characters has been debated among researchers since the emergence of reports on transport of genetic material between scion and rootstock (Pandey, 1976). Grafted plants were considered as "graft-chimera", involving tissues from two genetically different parents, till the introduction of "graft-hybrids" having genetic transformation in reproductive cells of scion by Pandey (1976). In his report, it was supposed that protoplast fusion and genetic transformation at the interface between scion and rootstock may possibly occur. In addition, possible activation of plant transposons and transpoisition of them in response to non specific graft induced changes (Zhang et al., 2002), and transport of exogenous DNA via the vascular system (Hirata et al., 1995) were also suggested. More recently, Stegemann and Bock (2009) have demonstrated that plant grafting could result in the exchange of genetic information via either large DNA pieces or entire plastid genomes.

The advantage of grafting, that it brings fruiting forward, makes the approach desirable for mass propagation of fruit crops for commercial benefit. However, it is important to determine the genetic stability of grafted plants since graft-induced changes may also lead to propagation of plants which have lower yield or tolerance and would therefore causing a commercial loss. Use of random amplified polymorphic DNA (RAPD) as genetic markers enables detection of genotypic alterations at the DNA level and has been widely used for genetic mapping, taxonomic and phylogenetic studies of many organisms (Selvi et al., 2008). Arbitrarily chosen primers are used to amplify a number of fragments from a DNA template to generate a discrete "fingerprint" when resolved by gel electrophoresis (Danylchenko and Sorochinsky, 2005). The prupose of the present study was the assessment of genetic stability or any graft-induced changes at molecular level in the tomato plants grafted on tobacco rootstock by RAPD analysis.

MATERIALS AND METHODS

Plant material

A Turkish cultivar of *Nicotiana tabacum* L. cv. Samsun was used as rootstock and a *Lycopersicon esculentum* Mill. commercial cv. H-2274 (MayAgro Seed Corporation, Turkey) was used as scion in grafting experiments.

Growth conditions

Tomato and tobacco plants were grown from seeds in greenhouse at 20-25ºC with a relative humidity of 45-55%. Seeds were sown into cell plants containing seedling substrate (Klasman-Deilmann GmbH, Germany) and grown for 50 and 20 days, respectively. Plants were transferred to soil in individual pots containing an animal based soil fertilizer (soil:fertilizer; 2:1) and grown for another 20-25 days in defined greenhouse conditions. The seeds of the scion were sown 30 days earlier than the seeds of the rootstock to ensure similar stem diameters at the time of grafting.

Grafting

Grafting of plants were performed in the plastic greenhouses at the Institute of Transplantation and Gene Sciences, Başkent University, Kazan-Ankara, Turkey in between May 17-19, 2009. Grafting of tomato plants on tobacco rootstocks were performed when tobacco plants had 6-7 and tomato plants had 3-4 real leaves. Tomato plants (denoted as T) were grafted on tobacco rootstocks (denoted as S) by cleft grafting method as previously described (Ersayın-Yaşınok et al., 2008). In brief, rootstock was cut down the middle of the stem at right angles with a sterile razor blade. The base of the scion was cut into a thin, narrow wedge and inserted into the cut of the rootstock. The graft union was covered with parafilm to enhance healing. Grafted plants (denoted as TS) were kept for 10 days under controlled conditions (24-26ºC with a relative humidity of 60-65% and a photoperiod of 16h (light)/ 8 (dark)). Grafted and non-grafted tomato plants were transplanted to soil field in greenhouse 60 cm apart from each other, and normal growth and cultural practices were followed for irrigation, fertilizer and pesticide applications.

Isolation of genomic DNA

Sixty days after transplanting (DAT) randomly sampled fresh leaves from 3 grafted and 3 non-grafted plants were collected. Genomic DNA was isolated by using NucleoSpin Plant (Macherey-Nagel, Austria) DNA isolation kit according to manufacturer's instructions. Quantity and purity of isolated DNA was spectrophotometrically determined by measuring optical density at 260 and 280 nm. Intactness of DNA was checked by native agarose (1% w/v) gel electrophoresis (90V, 1h) and visualized with ethidium bromide staining.

RAPD analysis

RAPD analysis of the DNA samples were performed using 10 commercial universal primers (Operon Technologies Inc., Alameda, CA, USA). Eight of these primers yielded reproducable and clear bands and chosen for further analysis (Table 1). Twenty five microliters of reaction volume contained 1X PCR buffer (MBI Fermentas, Lithuania) 2.5 mM $MgCl_2$ (MBI Fermentas), 0.2 mM dNTPs (MBI Fermentas), 0.2 µM primer, 200 ng of DNA and 1 unit of Taq DNA polymerase (MBI Fermentas). PCR conditions were; initial denaturation at 94ºC for 5 min, denaturation at 94ºC for 30 s, annealing at 33ºC for 75 s, extension at 72ºC for 90 s and a final extension at 72ºC for 5 min. Thirty five PCR cycles were performed in an Eppendorf Mastercycler Gradient (Eppendorf, UK) thermocycler. A negative control without genomic DNA was amplified in each run to ensure that no contaminated DNA was present in the reaction. PCR were performed as duplicates. PCR products were examined by native agarose (1.5% w/v) gel electrophoresis (80V, 85 min) and visualized with ethidium bromide staining using UVItec Platinum 2.0 GAS 7510 Acquisition Gel System (UVItec Limited, UK). RAPD profiles were analysed with UVIProplatinum_2.0 Image Acquisition and Analysis Software (UVItec Limited).

RAPD profiles and data evaluation

Evaluation of polymorphic RAPD profiles included determination of disappeared and appeared bands in comparison to control. Each alteration, determined and sized by the software, in banding pattern was given the arbitrary score of 1 where absence of polymorphic bands was given the score of 0 for each primer. Gain and/or loss alterations which do not occur in 3 all three samples from grafted plants were not considered in scoring. Polymorphism (P) and genomic template stability (GTS) was calculated by using RAPD scoring as follows:

$$P\ (\%) = [(a+b) / n] \times 100 \quad \text{[Equation 1]}$$

$$GTS\ (\%) = (1- [(a+b) / n]) \times 100 \quad \text{[Equation 2]}$$

where, a and b indicate appearance of new bands and disappearance of normal bands, respectively; n is the number of total bands in the control.

RESULTS

RAPD analysis and GTS of the grafted tomato plants

Eight primers gave a total of 102 bands in RAPD profiles of non-grafted tomato plants (Table 2). Two bands appeared and 1 band disappeared in RAPD profiles of all grafted plants with respect to non-grafted tomato plants (Figure 1). A 787 bp band was appeared in RAPD profile of all grfated plants obtained with primer 5 (Table 2) in a 475-1684 bp banding range when compared to non-grafted plants. RAPD profiles of grafted plants obtained with primer 7, had an appeared 786 bp band with respect to that of non-grafted plants in a 375-1700 bp banding range. In addition, a 821 bp band was disappeared in profiles of all grafted plants when compared to profiles of non-grafted plants. Considering total 3 polymorphic bands in a total of 102 bands obtained from 8 primers, polymorphism of the grafted tomato plants was caluculated as 2.94%. Concordantly, GTS was calculated as 97.06%.

DISCUSSION

Grafting is widely used for the improvement of agricultural yield and graft-induced changes have been reported in many cultivated plants, such as pepper (Taller et al., 1998), soybean (Hirata and Yagishita, 1986), and tomato (Hirata, 1980) in terms of changes in general characteristics, fruit shape, pungency, and plant type. In a study of Taller et al., (1998) it was reported that some phenotypic alterations were observed in the graft-induced variants of pepper and some characteristics of the stock were introduced into progeny. They have also demonstrated introduction of stable, new traits by grafting and suggested it as a novel genetic source in the breeding of pepper in another report (Taller et al., 1999). In a recent study of Stegemann and Bock (2009), two transgenic tobacco lines carrying different marker and reporter genes in different cellular components were grafted and the analysis of the graft sites revealed the frequent occurence of cells harboring both markers and reporters. Transport of either large DNA pieces or entire plastid genomes was suggested as a mechanism of genetic information exchange between stock and scion.

RAPD is a useful technique for the determination of genetic stability in ecotoxicology, genetics and plant breeding (Rout et al., 1998). Although reliability and reproducibility of RAPD assay have raised many questions (Ellsworth et al.,

1993), optimization of RAPD conditions have generated stable and reproducible results increasing its reliability (Atienzar et al., 1999; Becerril et al., 1999). Being a low cost and rapid technique, it allows studying large number of samples. Detection of polymorphisms and modifications of the genomic DNA is possible by comparison of RAPD profiles obtained by arbitrarily primed PCR. Basicly, disapperance of normal bands can be attributed to DNA lessions, such as bulky adducts, which reduce the number of DNA priming sites and have detrimental effects in a RAPD reaction. Appearance of new bands, on the other hand, can be result of variations (e.g. breaks, transpositions, deletions, etc.) in DNA structure (Danylchenko and Sorochinsky, 2005). Previously RAPD analysis was performed for the assessment of graft induced genetic variations and polymorphic banding patterns between RAPD profiles of grafted and non-grafted plants were determined (Taller et al., 1998; Chen and Wang, 2006).

In the present study, RAPD analysis was used to detect genetic variation between grafted and non-grafted plants. Eight primers revealed reproducible and clear band profiles and 2 primers among them generated specific markers in profiles of grafted plants. Appearance of amplified bands in profiles, obtained with primers 5 and 7, and disappearance of a normal band in profile, obtained with primer 7, were observed in RAPD analysis of the grafted plants. Since only 2 of the primers yielded graft-specific banding, altered bands constituted very low proportion of the total band number (Table 2). Genomic template stability of the grafted plants was calculated both from the RAPD analysis data obtained from each individual primer and by considering total polymorphic bands of the grafted plants in the total bands obtained from all primers. In the former one, average of the GTS values obtained from each individual primer was calculated. Calculated GTS values presented that both estimations of the GTS of grafted plants yielded similiar results (97.06% and 97.29%, respectively). Occurence of graft specific banding in only RAPD patterns of 2 primers out of 8 primers, suggested a low polymorphism in genomes of the grafts analyzed in the present study. In addition, no phenotypic variation, *e.g.* flowering time, growth of the plants, has been detected 60 DAT (data not shown). So, we evaluate all 8 RAPD profiles for the calculation of polymorphism and GTS percents in our experimental setup. Polymorphic bands in profiles of grafted plants may be due to transposition, most probably in non coding regions of DNA, caused by graft-induced stress rather than graft specific genetic exchange to scion.

There is a physiological effect of the rootstock on the scion, even there is not any genetic variation in scion. Transport of plant growth regulators, some proteins and RNAs across the graft union (Murfet, 1985; Zoubenko et al., 1994) have been reported. For example, Khah (2006) demonstrated that more tolerant root system of the rootstock may effect growth regulators in the scion of grafted plants faciliating water and nutrient uptake. Ohta (1991) well charecterized and differentiated two types of grafts; grafting for better growth and for genetic effect. In the first category the stock serves as a root system without branches and leaves of its own (as in our case), and in the later one stock serves as a host having many vigorous branches and leaves, and the scion growing on the stock as if parasitic without its own branches and leaves. In the same report, he also emphasized that no transmission of the genetic information would take place, unless translocation and integration of large functional DNA molecules, such as a gene, into new cell nuclei of the scion happened. Suggestions on genetic variation mechanism in grafts may be interpreted that graft induced genetic changes are not necessarily occuring in all grafts and/or with a high and stable frequency since it seems to be a random and occasional event. In addition, stability and transport of especially large DNA molecules may differ among different plant species depending on the action of nucleases. Furthermore, DNA changes in grafts may not necessarily cause phenotypic variations, unless these alterations cause functional changes at expression level. In case they cause phenotypic variations, inheritance of the gained trait should be introduced into the progeny for genetic transformation of the grafted plants.

In agricultural applications, high genomic stability of a succesive grafting may improve crop yield in a trait defined manner without involvement of genetic transformation. In this study, the genotypes of the grafted and non-grafted plants presented high genetic similarity based on the RAPD analysis, which was also supported by phenotypic observations. Supportive research on the issue using cytological analysis and analysis for the assessment of polymorphisms in progenies would further be conducted although results of the RAPD analysis provide a promising insight to subject.

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Table 1. Names and sequences of primers.

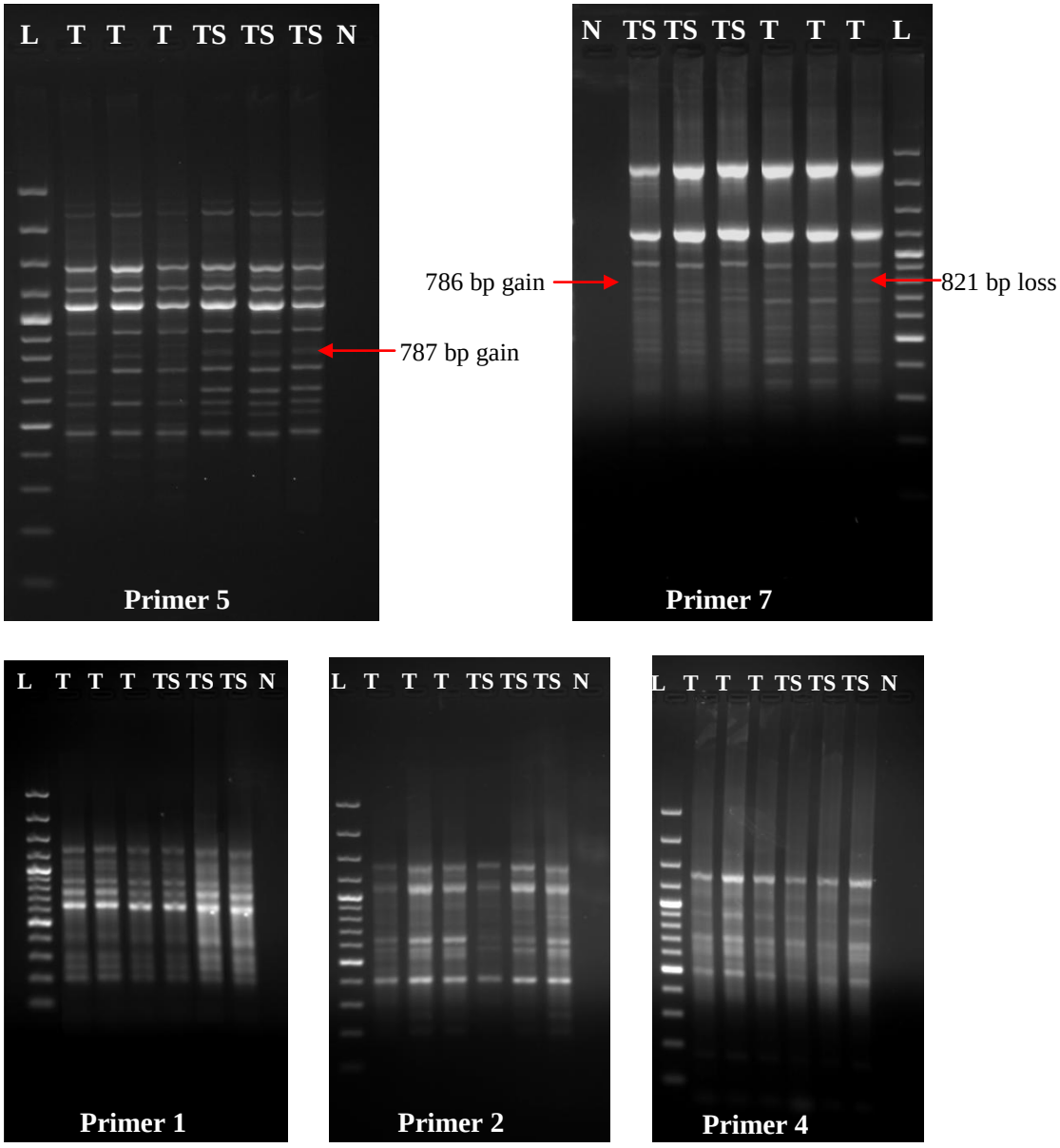
Primers	Name	Sequence (5'→ 3')
Primer 1	OPA-05	AGGGGTCTTG
Primer 2	OPA-09	GGGTAACGCC
Primer 3	OPA-13	AATCGGGCTC
Primer 4	OPA-16	AGCCAGCGAA
Primer 5	OPA-18	AGGTGACCGT
Primer 6	OPA-20	GTTGCGATCC
Primer 7	OPB-01	GTTTCGCTCC
Primer 8	OPC-01	TTCGAGCCAG

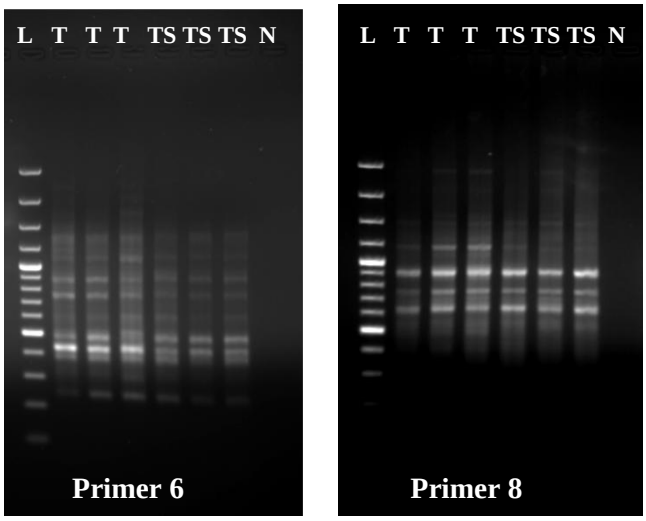
Table 2. RAPD analysis data of the grafted tomato plants with respect to non-grafted tomato plants and changes in GTS.

Primers	Range (bp [γ])	T	TS					GTS (%)	
			a[§]	Size (bp)	b[¥]	Size (bp)	a + b [†]	T	TS
Primer 1	202-1286	10	0	-	0	-	0	100	100
Primer 2	206-1364	14	0	-	0	-	0	100	100
Primer 3	313-1717	15	0	-	0	-	0	100	100
Primer 4	170-1277	10	0	-	0	-	0	100	100
Primer 5	475-2212	12	1	787	0	-	1	100	91.67
Primer 6	237-1412	12	0	-	0	-	0	100	100
Primer 7	337-2391	15	1	786	1	821	2	100	86.67
Primer 8	536-1522	14	0	-	0	-	0	100	100
Average								100	97.29
Total bands		102	2		1		3	100	97.06

[γ] base pairs
[§] appearance of new bands
[¥] disappearence of normal bands
[†] total polymorphic bands

Figure 1. RAPD profiles in the leaves of non-grafted tomato (T) and grafted tomato (TS) plants. N indicates negative control where L represents Gene Ruler™ 100 bp Plus DNA Ladder, (100-3000bp; MBI Fermentas).


L T T T TS TS TS N
787 bp gain
Primer 5
N TS TS TS T T T L
786 bp gain
821 bp loss
Primer 7
L T T T TS TS TS N
Primer 1
L T T T TS TS TS N
Primer 2
L T T T TS TS TS N
Primer 4


L T T T TS TS TS N
Primer 6
L T T T TS TS TS N
Primer 8