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in

Bellini E. (ed.), Giordani E. (ed.).
First Mediterranean symposium on persimmon

Zaragoza : CIHEAM
Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 51

2002
pages 81-86

Current situation of molecular markers for selecting pollination-constant and non astringent type from breeding populations of Japanese persimmon

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SUMMARY – In order to establish an effective breeding system of new pollination constant and non astringent (PCNA) type persimmons, we are seeking molecular markers which can distinguish PCNA-type in breeding populations at a juvenile stage. First, a bulked segregant analysis (BSA) using amplified fragment length polymorphism (AFLP) technique was used in a breeding population. From a total of 128 primer combinations, one AFLP marker was found to be linked to the dominant allele conferring the trait for astringency. Although this marker could distinguish about half of the individuals in the breeding population as non-PCNA-type, the remaining individuals of the population were mixed with PCNA- and non-PCNA-type. On the other hand, when we used this AFLP marker as a probe for Southern blot analysis, all non-PCNA-type individuals were distinguished from PCNA-type individuals by two polymorphic bands in the breeding population. Namely, by RFLP analysis of *Hin*d III-digested genomic DNAs, only non-PCNA-type individuals showed either a 6.5 or 8 kb band, or both by Southern blot analysis using the AFLP marker as a probe, whereas PCNA-type individuals had neither the 6.5 nor 8 kb band. Furthermore, when this RFLP analysis was applied to existing cultivars in our germplasm collection, the banding pattern clearly distinguished PCNA cultivars from non-PCNA cultivars. We are currently trying to design some primer pairs to detect these RFLP markers by PCR for practical use in the breeding system. In this paper, the results obtained from the trials to seek molecular markers for the selection of PCNA-type persimmons are reviewed.

Key words: AFLP marker, PCNA, selection, RFLP, astringency.

RESUME – ***"****Situation actuelle concernant les marqueurs moléculaires pour sélectionner le type à pollinisation constante et non astringent chez les populations de plaqueminier du Japon objet d'amélioration génétique". Afin de mettre sur pied un système efficace d'amélioration génétique d'un nouveau type de plaqueminiers à pollinisation constante et non astringents (PCNA), nous avons recherché des marqueurs moléculaires permettant de différencier le type PCNA chez les populations de sélection à un stade juvénile. D'abord, on a réalisé une analyse de ségrégation en masse (Bulk Segregant Analysis) en utilisant la technique AFLP chez une population de sélection. Sur un total de 128 combinaisons d'amorces, nous avons trouvé un marqueur AFLP qui était lié à l'allèle dominant conférant le caractère d'astringence. Bien que ce marqueur permette de différencier environ la moitié des individus dans la population de sélection en tant que type non PCNA, les individus restants de la population étaient mélangés avec les types PCNA et non PCNA. D'autre part, lorsque nous avons utilisé ce marqueur AFLP comme sonde pour l'analyse southern blot, tous les individus de type non PCNA ont été différenciés des individus de type PCNA par deux bandes polymorphiques chez la population de sélection. A savoir, par analyse RFLP d'ADN génomiques digérés par* Hind *III, seulement les individus de type non PCNA ont montré une bande soit 6,5 soit 8 kb, ou toutes deux, par analyse southern blot en utilisant le marqueur AFLP comme sonde, tandis que les individus de type PCNA n'avaient ni la bande 6,5 ni la bande 8 kb. En plus, lorsque cette analyse RFLP a été appliquée aux cultivars existants dans notre collection de germoplasme, la configuration des bandes a permis de différencier clairement les cultivars PCNA des cultivars non PCNA. Actuellement nous essayons de mettre au point des paires d'amorces pour détecter ces marqueurs RFLP par PCR pour les utiliser dans la pratique dans le système d'amélioration génétique. Dans cet article sont passés en revue les résultats obtenus à partir des essais recherchant des marqueurs moléculaires pour la sélection de plaqueminiers de type PCNA.*

Mots-clés : *Marqueur AFLP, PCNA, sélection, RFLP, astringence.*

Introduction

Japanese persimmon (*Diospyros kaki*) cultivars are classified into four types depending on the nature of astringency-loss: (i) pollination-constant and non astringent (PCNA); (ii) pollination-variant and non astringent (PVNA); (iii) pollination-variant and astringent (PVA); and (iv) pollination-constant

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and astringent (PCA). Among these four types, PCNA-type is the most desirable for fresh fruit consumption due to its stable astringency-loss on the tree. So far, the breeding objectives for persimmon have been focused on obtaining PCNA-type offspring with better eating qualities and early maturation.

The trait of natural astringency-loss in PCNA-type is qualitatively inherited and is recessive to the other three types (non-PCNA-type). This means that the crossing between PCNA- and non-PCNA-type yields only non-PCNA-type offspring in F_1 progenies. To yield PCNA type, these F_1 plants have to be backcrossed to PCNA-type. But this backcrossed population yields only around 15% of PCNA type due to the polyploid nature of Japanese persimmon. So, crossings for breeding of PCNA-type have been restricted among PCNA cultivars and/or selections for obtaining PCNA-type in F_1 generation. However, the genetic diversity of PCNA cultivars and/or selection is very narrow due to their recent origin. More than 1000 persimmon cultivars are reported to exist in Japan, but only six cultivars have been described as PCNA-type (Agricultural Research Station, 1912). Currently, inbreeding depression is a serious problem for breeding populations of PCNA-type and the introduction of non-PCNA-type in breeding projects becomes necessary to spread the genetic base of breeding populations despite the need of two generations to obtain PCNA-type offspring (Yonemori *et al.*, 2000).

Due to these situations for breeding new PCNA cultivars, we need to establish an effective system for the selection of PCNA-type individuals in breeding populations at a juvenile stage. Hence, we have started looking for molecular markers to distinguish PCNA-type in the breeding population and have found a very reliable candidate for it. In this present paper, we will review the current situation of our research on the molecular marker in the breeding population and show its effectiveness in discriminating PCNA-type individuals.

Materials and methods

Bulked segregant analysis (BSA) by amplified fragment length polymorphism (AFLP)

Total DNAs were extracted from the leaves of 6 PCNA-type and 10 non-PCNA-type plants from the progeny crossed between 18-14 and 170-26 (Fig. 1). The procedures for DNA extraction of Doyle and Doyle (1987) were followed, and the extracted DNAs from 6 PCNA-type or 10 non-PCNA-type plants were pooled as a PCNA bulk or a non-PCNA bulk, respectively, for BSA (Michelmore *et al.*, 1991).

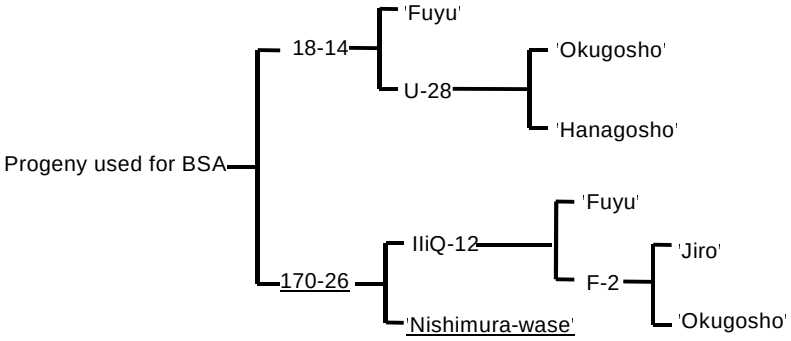

Fig. 1. Pedigree of the progeny used for BSA. The non PCNA type cultivar/selection is underlined. The others are PCNA type cultivars/selections.

AFLP analysis was performed by AFLP analysis system I kit (Life Technologies, Md., USA). Briefly, the bulked DNAs were digested with *Eco*RI and *Mse*I, and ligated *Eco*RI and *Mse*I adapters. Then, pre-selective and selective amplification were performed with two primers based on the sequence of *Eco*RI and *Mse*I adapters including one or three additional selective nucleotides at the 3' end of each primer.

After selective amplification, PCR products were denatured and aliquots of each reaction were loaded onto denaturing 6% polyacrylamide gel. Then, after running the gel, the amplified fragments were transferred to a Biodyne B membrane (Pall, Inc., NY, USA) and hybridized with an AFLP non radioactive probe (Life Technologies). The fragments were finally detected to incubate with CDP-Star (Boehringer Mannheim, Ind., USA) at 37°C for 15 min and exposed to X-ray film for 3 to 7 h.

Southern blot analysis in backcrossed progenies

After sequencing of the most reliable AFLP marker, the marker was used as a probe for Southern blot analysis on the backcrossed progeny used for BSA and the individuals from different breeding progenies. In total, 42 individuals were used for this analysis. For Southern blot analysis, 20 µg of total DNAs were digested with *Hin*dIII and run on 0.8% agarose gel, transferred to a nylon membrane (Hybond-N; Amersham, Buckinghamshire, UK), and hybridized with the DIG-labelled probe at 65°C for 16 h. After high stringency washes, the bands were detected immunologically using the anti-DIG-alkaline phosphatase conjugate and the chemiluminescent substrate CDP-Star.

Effectiveness of RFLP analysis on existing cultivars

RFLP analysis was performed on existing cultivars in the germplasm collection of our university. Thirteen PCNA and twenty non-PCNA cultivars were analyzed for evaluation of RFLP analysis to distinguish PCNA-type from non-PCNA-type. The extraction of total DNA and Southern blot analysis were performed by the same procedures as described above.

Design of some primer pairs for detection of RFLP markers by PCR

Based on partial sequence of the two RFLP markers, we have designed a couple of primer pairs to amplify a part of the regions of two RFLP markers which were detected by Southern blot analysis using the AFLP marker as a probe after digestion of total DNA with *Hin*dIII. The individuals used for BSA were examined for this PCR analysis using the designed primer pairs.

Results and discussions

Detection of AFLP marker from BSA and applying it to Southern blot analysis

A total of 128 primer combinations of 8 *Eco*RI and 16 *Mse*I primers were tested in a BSA against subsets of PCNA and non-PCNA bulks. About 40 bands were detected per primer combination and 56 polymorphic markers were identified between two bulks. These makers were absent in PCNA bulk and present in non-PCNA bulk. However, after these 56 candidate markers were examined against the individuals used for BSA, only one marker was chosen as the reliable marker (Fig. 2). This reliable marker was recovered from the gel and sequenced. Based on the sequence, we made a pair of primers to detect sequence characterized amplified region (SCAR) of non-PCNA-type individuals for discriminating it from PCNA-type individuals. But, PCR analysis of this primer pair revealed no polymorphisms between PCNA- and non-PCNA type (Fig. 3).

On the other hand, when this AFLP marker was used as a probe for Southern blot analysis on *Hin*dIII-digested genomic DNA, all 10 non-PCNA-type individuals used for BSA showed the band of 6.5 or 8 kb, or both, whereas the 6 PCNA-type individuals for BSA had neither of the bands (Fig. 4). This result clearly shows that we can select PCNA-type in the breeding progeny by RFLP analysis using the AFLP marker as a probe. In addition, the segregation pattern of the 6.5 kb band was the same as that of the AFLP marker obtained by AFLP analysis in the breeding population used for BSA (Table 1). This indicates that the 6.5 kb RFLP marker is linked to the same dominant allele as the AFLP marker, and that the other 8 kb RFLP marker is linked to another dominant allele.

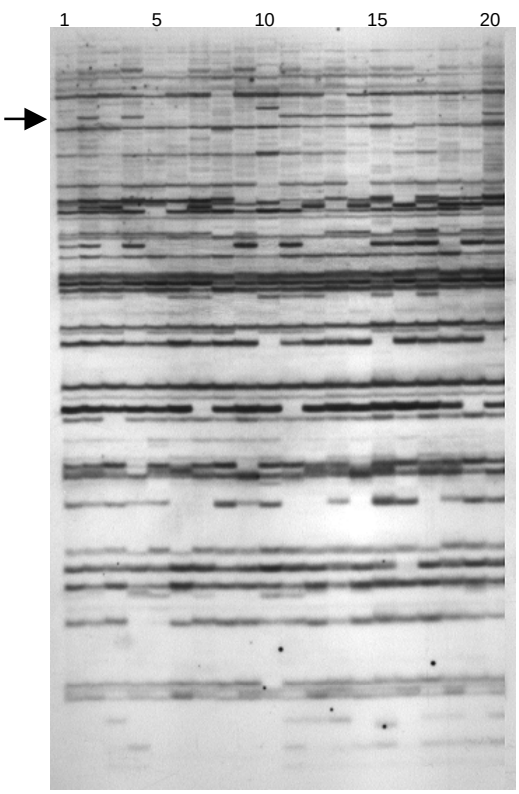

Fig. 2. AFLP fingerprints of 16 progenies used for bulked segregant analysis (BSA), generated using primer combination E-ACC/M-CTA. *Lane 1*: PCNA bulk; *2*: non-PCNA bulk; *3*: 18-14 (PCNA parent); *4*: 170-26 (non-PCNA parent). Arrow indicates the reliable AFLP marker.

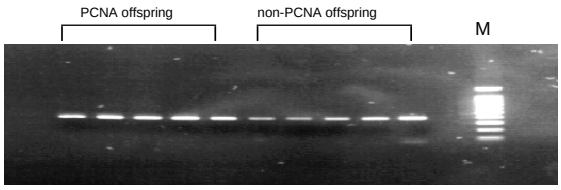

Fig. 3. Amplification of genomic DNA of 10 individuals used for BSA by primers designed for the detection of the AFLP marker. M : 100 bp DNA ladder.

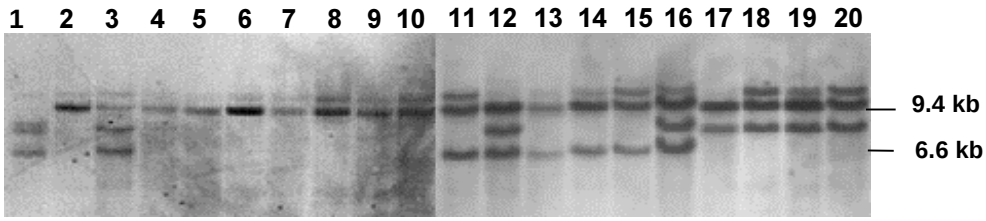

Fig. 4. RFLP analysis of 16 offspring used for BSA and their parents. *Lane 1*: Nishimurawase (non-PCNA parent); *2*: IIiQ-12 (PCNA parent); *3*: 170-26; *4*: 18-14; *5-10*: PCNA genotypes; *11-20*: non-PCNA genotypes.

Table 1. Presence (+) or absence (–) of AFLP marker and RFLP marker in the progenies used for bulked segregant analysis

Maker	PCNA genotype						Non-PCNA genotype									
	1	2	3	4	5	6	1	2	3	4	5	6	7	8	9	10
AFLP	–	–	–	–	–	–	+	+	+	+	+	+	–	–	–	–
RFLP 6.5 kb	–	–	–	–	–	–	+	+	+	+	+	+	–	–	–	–
RFLP 8 kb	–	–	–	–	–	–	–	+	–	–	–	+	+	+	+	+

This RFLP analysis was also effective in discriminating PCNA-type from non-PCNA-type in the progenies from other breeding populations. We examined a total of 26 individuals, in which 13 individuals were PCNA-type and 13 remaining individuals were non-PCNA-type, and revealed that all PCNA-type individuals had neither the 6.5 or 8 kb band, whereas non-PCNA-type individuals had either a 6.5 or 8 kb band, or both. Therefore, based on the RFLP pattern, we could separate PCNA-type offspring in the breeding progenies with 100% accuracy.

Effectiveness of RFLP analysis on existing cultivars

RFLP analysis on *Hin*dIII-digested genomic DNA using the AFLP marker as a probe was also effective to discriminate PCNA cultivars from non-PCNA cultivars. Thirteen PCNA cultivars showed no polymorphisms in banding pattern as shown in the above results of breeding population, except for one Chinese cultivar 'Lou Tian Tian Shi' (Fig. 5).

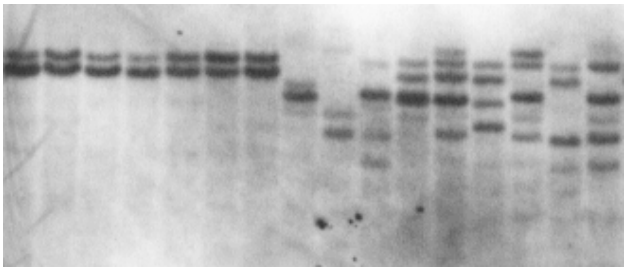

Fig. 5. RFLP analysis in some persimmon cultivars. Lanes 1-8 are PCNA cultivars; 1: Fuyu, 2: Jiro, 3: Hana-gosho, 4: Oku-gosho, 5: Gosho, 6: Fujiwara-gosho, 7: Yamato-gosho, 8: Luo Tian Tian Shi. Lanes 9-16 are non-PCNA cultivars; 9: Shougatsu, 10: Saijo, 11: Aizumishirazu, 12: Atago, 13: Tenryubou, 14: Kikuhira, 15: Yokono, 16: Amayotsumizo.

On the contrary, all 20 non-PCNA cultivars had many polymorphic bands (Fig. 5). Based on the banding pattern of RFLP analysis, PCNA cultivars could be distinguished from non-PCNA cultivars, except for 'Lou Tian Tian Shi'.

The Chinese cultivar 'Lou Tian Tian Shi' is reported to be distantly related to Japanese PCNA cultivars (Kanzaki *et al.*, 2000). The mechanisms to become PCNA-type on 'Lou Tian Tian Shi' may be different from PCNA cultivar of Japanese origin.

PCR analysis by designed primer pairs from RFLP markers

We have designed some primer pairs from partial sequencing of two RFLP markers (6.5 kb and 8 kb). The primer pair was designed for amplifying specific regions of 6.5 kb or 8 kb of RFLP marker. The effectiveness of these primer pairs is being evaluated in the breeding population used for BSA. We are now receiving the results from this evaluation and they indicate the good efficiency of the designed primer pair.

Conclusion

To select PCNA-type individuals in breeding populations at a juvenile stage is a dream for most persimmon breeders. Our trials to find a molecular marker could make it a reality in the near future. The RFLP technique should be applicable and effective to most breeding populations and a marker-assisted breeding system for selecting PCNA-type persimmon is just around the corner for practical use.

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