การพัฒนาเครื่องหมายโมเลกุลแบบเอเอฟแอลพีเพื่อการตรวจสอบสายพันธุ์ยาสูบ

Development of AFLP Molecular Marker for Variety Identification of Tobacco Leaves

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บทคัดย่อ ยาสูบเป็นพืชที่มีความสำคัญทางเศรษฐกิจของประเทศไทย ปัจจุบันวิธีการที่ใช้จำแนกสายพันธุ์ยาสูบยังไม่มีความแม่นยำเพียงพอ ในการศึกษาครั้งนี้จึงได้นำเอาเทคนิคเอเอฟแอลพีมาใช้กับใบยาสูบสด ในการศึกษาความแตกต่างทางพันธุกรรมระหว่างสายพันธุ์ยาสูบที่ปลูกในประเทศไทยทั้งสายพันธุ์นำเข้าและสายพันธุ์พื้นเมือง จากแผนภูมิแสดงความสัมพันธ์ทางพันธุกรรมทั้งแบบเอ็นเจและยูพีจีเอ็มเอ ให้ผลการจัดกลุ่มพันธุ์ยาสูบที่คล้ายกัน โดยที่สายพันธุ์นำเข้าเกือบทั้งหมดถูกจัดกลุ่มอยู่ด้วยกัน อย่างไรก็ตาม ยาสูบสายพันธุ์นำเข้า 3 สายพันธุ์ (Coker326, Samsun และ Xanthiyaka) ได้ถูกจัดรวมกลุ่มร่วมกับยาสูบสายพันธุ์พื้นเมือง จากการศึกษาเบื้องต้นนี้แสดงให้เห็นว่าเครื่องหมายโมเลกุลเอเอฟแอลพีมีศักยภาพสำหรับการพัฒนาเป็นเครื่องมือสำหรับใช้ศึกษาความแตกต่างทางพันธุกรรมระหว่างสายพันธุ์ยาสูบ ในขณะนี้กำลังอยู่ในระหว่างการปรับปรุงปฏิกิริยาพีซีอาร์ ให้มีความเหมาะสมในการศึกษาตัวอย่างใบยาสูบบ่มแห้ง

Abstract Tobacco is one of important economic crops in Thailand. Nowaday, current approaches to distinguish tobacco varieties are not accurate enough. In this study AFLP technique performed with fresh leaf specimen was introduced to investigate genetic differences of tobacco cultivated in Thailand, both imported and local varieties. Genetic relationship trees based on NJ and UPGMA techniques revealed similar clusterings. Almost all imported varieties were clustered together. However, 3 imported varieties (Coker326, Samsun and Xanthiyaka) were grouped with the local varieties. This preliminary study suggested that AFLP molecular marker method has potential to be further developed as tool for the study of genetic differences between tobacco varieties. At the moment, we are improving the PCR condition which would be suitable for dry-cured leaf samples.

Keywords: AFLP, molecular marker, Thailand, tobacco, variety identification

Introduction

Tobacco has been cultivated for thousands of years and has served as a raw material for cigarette and cigar industries of many countries. Almost all of the commercial tobaccos produced in the world are *Nicotiana tabacum*. It is also one of the most important crops of Thailand. Tobacco was first introduced into Thailand in the 16th century. By Thai law, all tobacco varieties cultivated in Thailand are defined to two variety groups: local varieties and imported (Virginia, Burley & Turkish) varieties. This variety separation leads to some differences in tariff-collecting and crop-growing regulations between the local and imported tobacco varieties. Moreover, if tobacco leaves from either of the two variety groups are dry-cured, there would not be any accurate approach to distinguish whether the leaves are of the local or imported varieties. From such problem, some molecular markers have been introduced to determine genetic differences between tobacco varieties (for example, Del Piano et al., 2000; Rossi et al., 2001; Zhang et al., 2006). Molecular markers (or genetic markers) have become useful tools to provide a relatively unbiased estimation of genetic diversity in plants (Clegg, 1997). The genetic relationship studies of Thai tobacco varieties are so important because there have not been such genetic information available before even though many different commercial varieties were developed in Thailand. Recently, our group has successfully developed ISSR (Inter-Simple Sequence Repeat) molecular markers to distinguish some local tobacco varieties cultivated in Thailand (Denduangboripant et al., 2008). However, these ISSR makers were unfortunately suitable only for fresh leaf samples, not dry-cured tobacco leaves.

AFLP (Amplified Fragment Length Polymorphism) is another molecular marker which has been successfully used in polymorphism analysis, crop cultivar identification, and phylogenetic evaluation. The AFLP technique is also a favourable method for cultivated crops like tobacco cultivars in which the genetic distances are too small for other molecular marker techniques. The AFLP technique is based on the PCR (Polymerase Chain Reaction) amplification of a subset of genomic restriction fragments. The AFLP system is composed of four successive enzymatic-reaction steps: digestion of DNA with restriction endonucleases, ligation of two specific adapters to the restriction fragments, pre-selective PCR amplification, and selective PCR amplification. The reason why AFLP has been extensively used in genetic diversity analyses of crop plants is its maximum coverage of the whole-genome in a short time. Generally, AFLP produced more polymorphic loci per primer in any diversity study than RFLP, SSR or RAPD techniques (Bogani et al., 1997).

Previously, Rossi et al. (2001) studied AFLP markers of tobaccos and found that AFLP appeared to be an appropriate technique for genetic fingerprinting of both fresh and processed tobacco leaves. Another AFLP analysis of genetic polymorplisms and evolutionary relationships among cultivated and wild *Nicotiana* species showed that the genetic polymorphism presenting among cultivated tobacco lines (*N. tabacum*) was limited (Ren and Timko, 2001). In addition, AFLP genetic-diversity study among flue-cured tobacco (*N. tabacum*) revealed that the flue-cured tobacco germplams commonly grown in China have narrow genetic diversity among the cultivars (Zhang et al., 2006). In 2008, Siva et al. studied genetic polymorphism of Indian tobaccos using AFLP and found that the cultivated flue-cured varieties were clustered separately from the air-cured type. The AFLP markers of Siva et al. were also found being specific to some true hybrid varieties such that they can be used in a genotypic identification in trades and commerces. From these reviewed literatures and the fact that no genetic analysis of Thai tobacco varieties using AFLP has been done before, in this study we then introduced AFLP molecular marker technique to examine genetic differences between local and imported tobacco varieties grown in Thailand.

Materials and Methods

Plant materials

Leaf specimens of 9 imported and 13 local varieties were sampled in the crop field from 10 different provinces around Thailand with help of Thailand Tobacco Monopoly, Ministry of Finance. Additionally, three imported and five local varieties were obtained from the cultivating greenhouse of Maejo Tobacco Experiment Station, Chiang Mai province. All imported tobacco varieties were separated into three sub-varieties (Virginia, Burley and Turkish). These varieties were classified by methods of curing. (Virginia: flue curing, Burley: light air curing, and Turkish: sun curing). The tobacco leaves were kept separately in silica gel bags and stored at room temperature until used for genomic DNA preparation.

Genomic DNA Extraction

The leaf samples were ground to fine powder with liquid nitrogen and total genomic DNA was extracted using Plant Genomic DNA Mini kit (Geneaid, Taiwan) following an instruction of the manufacturer. The concentration of the extracted DNA was estimated on 0.8% agarose gel electrophoresis using 100 bp ladder as a standard DNA marker. The extracted genomic DNA was maintained at -20^{o}C until used.

PCR-AFLP marker

The AFLP marker amplification was performed based on the protocol of Vos et al. (1995) with some modification. The extracted genomic DNA (approximately 250 ng) was digested completely with *Eco*RI and *Tru*9I restriction enzymes in a total volume of 25 µl. *Tru*9I is an isoschizomer of *Mse*I. *Eco*RI and *Mse*I oligonucleotide adapters were subsequently ligated to the digested DNA fragments. *Eco*RI primers were 5’-GAC TGC GTA CCA ATT C-3’ and MseI primers were 5’-GAT GAG TCC TGA

GTA A-3'. The pre-amplification step was first carried out using adapter-specific primers with a single selective nucleotide on each primer: *Eco*RI primer (E_A) and *Mse*I primer (M_C). The pre-selective amplification using the following cycling parameters: 20 cycles of 30 s at 94ºC, 60 s at 56ºC, 60 s at 72ºC. The pre-amplified DNA was used as a template for selective amplification using +3 primers of *Eco*RI and *Mse*I adapters. On this step, thirty-two selective AFLP primer-pairs were screened whether any of them could produce polymorphic band from "all" tobacco samples. The cycling parameters of this step were: the first cycle of 30 s at 94ºC, 30 s at 65ºC and 60 s at 72ºC, and lowering the annealing temperature by 0.7ºC per cycle for other 11 cycles, followed by 23 cycles of 94ºC for 30 s, 56ºC for 30 s and 72ºC for 60 s. The AFLP amplified products were separeted by electrophoresis on 6% denaturing polyacrylamide gel containing 7M urea. The separated AFLP-PCR products were visualised by silver staining. Sizes of the fragments were estimated using 50 bp and 100 bp DNA ladder markers.

AFLP Data analysis

Only the bright, clearly-resolved AFLP fragments generated from each primer combination were scored for presence (1) or absence (0) of the bands of the 30 tobacco varieties. Nei and Li's coefficient analysis (1979) was used to calculate pairwise band similarity values of the samples using program PAUP* 4.0b10. Cluster analysis and dendrogram construction were performed using Unweighted Pair Group Method of Arithmatric Mean (UPGMA) and Neighbor-Joining (NJ) methods. Reliability of the clusters was estimated by bootstrap analysis with 10,000 replications.

Results

AFLP-PCR amplification

From our preliminary AFLP study on 30 tobacco varieties (12 imported varieties and 18 local varieties), thirty-two selective AFLP primer-pairs were screened against all tobacco samples. Only four (E_{AAG}/M_{CAA}, E_{AAG}/M_{CAT}, E_{AAG}/M_{CGC} and E_{AAG}/M_{CGC}) could be suitable used as +3 primers for the selective AFLP-PCR step. The fingerprinting results provided a total number of 2,592 DNA fragments from the combination of four the primers with an average of 279.75 polymorphic loci per primer. There were totally 1,119 (43.17%) amplified polymorphic bands and 1473 (56.83%) monomorphic bands (Table 2). The average polymorphic percentage was 59.47%.

The sizes of bands amplified by each primer ranged from 150 bp to 700 bp, with an average of 648 fragments per primer combination. The highest number of the amplified fragments was by the primer pair E_{AAG}/M_{CGC} (1,132 bands), also having fewer smeared background than the others (Fig.1). The lowest number of the amplified fragments was by the E_{AAG}/M_{CAA} pair (159 bands). The primer combination E_{AAG}/M_{CAA} gave the highest polymorphism (80.50%) across all varieties, whereas the E_{AAG}/M_{CGC} pair gave the lowest (36.40%) score.

Genetic relationship analyses

Neighbour-Joining (NJ) and UPGMA genetic relationship trees were derived from whole AFLP data with Nei and Li's similarity coefficients. The NJ tree (Fig. 2a) showed four groups of tobacco varieties which had bootstrap values higher than 50%.

Table 1 Tobacco varieties used in this study.

Variety name	Variety group	Area of collection (province)
K187	Imported(Virginia)	Nakhon Phanom
K326	Imported(Virginia)	Lamphun
PVH03	Imported(Virginia)	Phayao
PV09	Imported(Virginia)	Chiang Rai
Coker326	Imported (Burley)	Phrae
B1 special	Imported (Burley)	Sukhothai
KY14	Imported (Burley)	Sukhothai
Samsun	Imported(Turkish)	Nakhon Phanom
Xantiyaka	Imported(Turkish)	Nakhon Phanom
HBO04P	Imported (Burley)	Maejo Tobacco Experiment Station, ChiangMai

Table 1 Cont.

Variety name	Variety group	Area of collection (province)
TN90	Imported (Burley)	Maejo Tobacco Experiment Station, Chiang Mai
TN 97	Imported (Burley)	Maejo Tobacco Experiment Station, Chiang Mai
Yamueang	Local	Phayao
K382-phuenmueang	Local	Nong Khai
Kariang	Local	Kanchanaburi
Kan	Local	Suphan Buri
Kan-kiw Dok-chom-phu	Local	Suphan Buri
Kan-kiw Dok-khao	Local	Suphan Buri
Laodong	Local	Kanchanaburi
Meao	Local	Nakhon Phanom
Hangkai	Local	Kanchanaburi
Phu	Local	Nong Khai
Yahan	Local	Nakhon Phanom
Petkhangsing	Local	Sukhothai
Petmakhuea	Local	Sukhothai
Padang	Local	Maejo Tobacco Experiment Station, Chiang Mai
Pasak	Local	Maejo Tobacco Experiment Station, Chiang Mai
Linchang	Local	Maejo Tobacco Experiment Station, Chiang Mai
Chorlare	Local	Maejo Tobacco Experiment Station, Chiang Mai
Nisan	Local	Maejo Tobacco Experiment Station, Chiang Mai

Table 2 The number of bands and degrees of polymorphism revealed by four AFLP primer combinations.

Primer combination	Total band	Polymorphic band	Polymorphic percentage (%)
E_{AAG}/M_{CAA}	159	128	80.50
E_{AAG}/M_{CAT}	772	383	49.61
E_{AAG}/M_{CGC}	1,132	412	36.40
E_{ACT}/M_{CAG}	529	288	54.44
Total	2,592	1,119	59.47
Average	648	279.75	

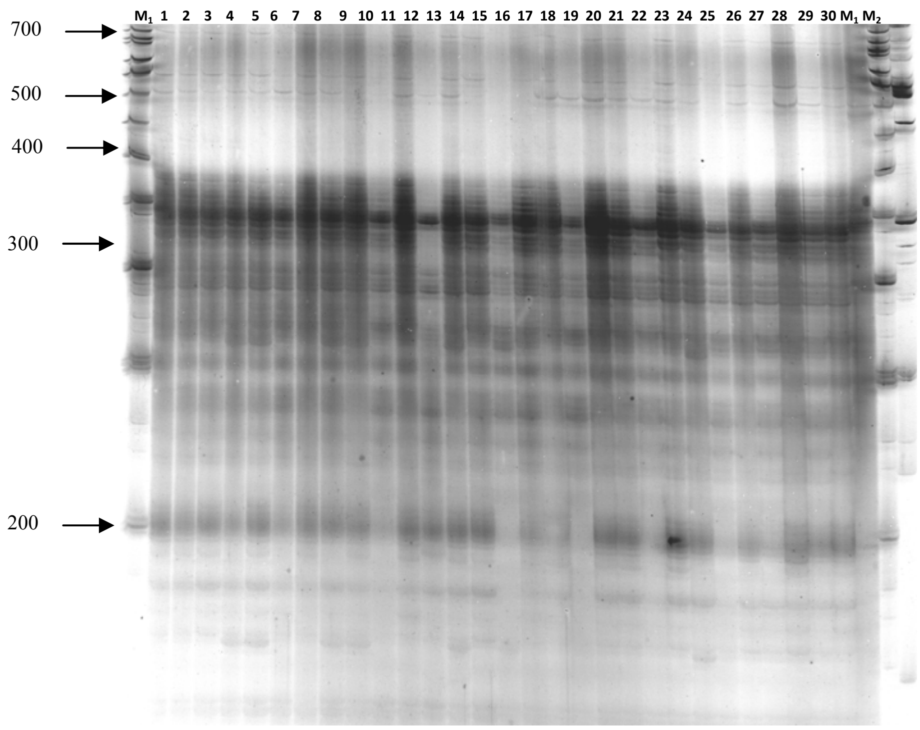

Figure 1 An AFLP pattern of 30 tobacco variety samples using E_{AAG}/M_{CGC} selective primers (lane no. 1-30 = tobacco varieties : K187, K326, PVH03, PV09, B1.sp, KY14, HBO04P, TN90, TN97, Samsun, Xanthiyaka, Coker326, Chorlare, Nisan, Pasak, Padang, Petkhangsink, Petmakhua, Yamuang, Linchang, Hangkai, Phu, Yahun, K326 Phun-mueang, Kariang, Kan, Kan-kiw Dok-chom-phu, Kan-kiw Dok-khao, Laodong, and Maeo, respectively. 50 bp and 100 bp DNA ladder markers (lane M_1 and M_2) were used).

Group I included PVH03, PV09 and B1.sp with 81% bootstrap support. In Group II, PV09 and B1.sp varieties were paired together with 53% bootstrap value while Xanthiyaka and Coker326 were grouped together in Group III with high bootstrap percentage (98% bootstrap). HBO04P and TN97 were paired as Group IV with 51% bootstrap and the other groupings of the other varieties left had lower than 50% bootstrap support.

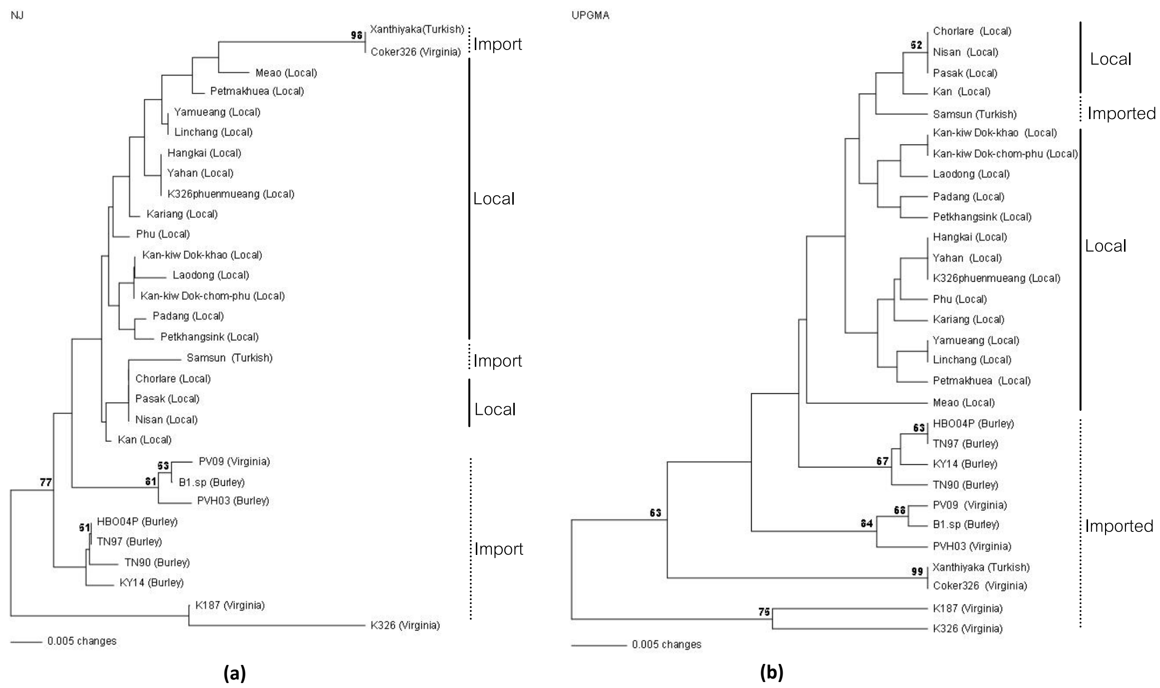

Figure 2 Genetic relationship trees from total AFLP bands of 30 tobacco samples based on Nei and Li's similarity coefficient using (a) Neighbour-joining (NJ) and (b) UPGMA methods. Numbers along branches are bootstrap-supporting values generated after 10,000 replications. The bootstrap values having less than 50% are not shown on the trees.

The UPGMA tree revealed seven groups with higher than 50% bootstrap values. Group I revealed the pair of K187 and K326 varieties with 75% bootstrap value. Group II consisted of PVH03, PV09 and B1.sp with 84% support. Group III contained PV09 and B1.sp with 68% bootstrap while KY14, HBO04P, TN90, and TN97 were grouped together as Group IV with 67% bootstrap support. Group V paired HBO04P and TN97 with 53% bootstrap whereas Chorlare, Nisan and Pasak were clustered together as Group VI with 62% value. The strong grouping of Xanthiyaka and Corker326 with 99% bootstrap support was named Group VII.

Discussion and Conclusions

Our preliminary AFLP study showed that four selective primer pairs (E_{AAG}/M_{CAA}, E_{AAG}/M_{CAT}, E_{AAG}/M_{CGC} and E_{ACT}/M_{CAG}) gave clearer and amplified bands than other tested primers. This AFLP result also revealed a large number of scorable polymorphic fragments per primer (see an example in Fig. 1). A total of 2,592 AFLP bands were obtained from all 30 tobacco varieties amplified with the four primers. The average number of AFLP fragments per primer in this study was 648 fragments. Not only better than RAPD, our AFLP study gave higher number of bands than a previous AFLP report of cultivated tobacco accessions (Ren and Timko, 2001), only 92 bands. The RAPD study of Del Piano et al. (2000) could detect 106 RAPD fragments in 20 tobacco lines, or only 8.15 fragments per primer. In addition, Siva et al. (2008) and Zhang et al. (2006) produced 107.4 and 249 AFLP-PCR fragments of tobacco varieties per primer, respectively. This clearly indicates the power of the AFLP analysis. Although our study could give high number of amplified AFLP fragments, the four primer-pairs generated only 1,119 polymorphic bands (43.17%), or an average of 59.47% in each primer which is less than the result of Siva et al (2008).

The NJ and UPGMA trees revealed genetic relationships among 30 tobacco varieties that all local varieties were closely related and clustered together in both trees. This grouping of the local varieties was not based on their cultivated areas. Only imported varieties were also grouped together except Samsun (of Turkish varieties group) which was clustered with Chorlare, Kan, Pasak and Nisan local varieties. Our finding does not agree well with some previous studies. For example, cultured tobacco groups could be divided primarily based on geographic origin and manufacturing quality traits (Ren and Timko, 2001). The polymorphism among cultivated tobacco lines was also limited by the high degree of similarity.

In conclusion, the AFLP-PCR molecular marker used in this study could mostly determine the genetic difference between local and imported tobacco varieties from fresh leaf specimen. Thus, this AFLP approach should be further developed to be an effective molecular marker for dry-cured leaf samples to distinguish long-cultivated local varieties from the imported varieties grown in Thailand.

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