African Journal of Biotechnology Vol. 11(33), pp. 8196-8201, 24 April, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.243
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Identification of a male-specific amplified fragment length polymorphism (AFLP) marker in *Broussonetia papyrifera*

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Accepted 2 April, 2012

The present study exhibits amplified fragment length polymorphism (AFLP) molecular marker for sex identification in *Broussonetia papyrifera*. Based on nine selective amplification primer combinations, 230 bands were produced and the *E*-AGG/M-CAA combination was found to be a male-specific AFLP marker. Subsequently, this male-specific AFLP fragment was sequenced and converted into a sequence tagged site (STS) marker. Based on STS sequence, two primers, MADB-1 and MADB-2 (Male-Associated DNA from *B. papyrifera*), were designed to verify the specificity of the fragment. The results indicate that common homology sequence is existed in both male and female plants while one of the bands amplified via MADB-2 primer was solely present in male individuals at high annealing temperature up to 66°C. Finally, MADB-2 primer was introduced to amplify another 16 plants and it revealed that this primer could be used as a convenient, efficient, reliable, and low-cost molecular marker for sex identification in *B. papyrifera.*

Key words: *Broussonetia papyrifera*, AFLP, STS, male-specific marker.

INTRODUCTION

Broussonetia papyrifera (Moraceae) is a dioecious plant and its fruits have been widely used in traditional medicine for the treatment of impotence, age-related disorders, ophthalmic disorders, and so on (Lee et al., 2001; Mei et al., 2009; Zheng et al., 2008). The bark is composed of very strong fibers, and has been used for manufacturing high-quality papers, clothes, and ropes while the leave can be eaten and used for animal fodder (Whistler and Elevitch, 2006). However, *B. papyrifera* is considered to be an invasive pest when introduced into new areas. The plant is known to quickly disrupt native habitats, become highly invasive and upset natural ecosystems (Malik and Husain 2007; Nagpal et al., 2011). To avoid the species invasion, only male *B.papyrifera* were introduced into the pacific islands, subsequently, the invasion was blocked owing to absence of female viable seeds in the Pacific (Whistler and Elevitch 2006). By now, no recorded evidence of any monoecious genotypes was found to be existed in *B. papyrifera* species (Coder, 2008). Moreover, both male and female species are morphologically alike and hare to distinguish in sterile state (Whistler and Elevitch, 2006). It has been reported that female flowers possess a two- to four-lobed perianth and a superior ovary with a filiform style, which are remarkably different from male flowers. Unfortunately, the flowering process of most *B. papyrifera* species is unknown and of infrequent occurrence (Whistler and Elevitch, 2006). Thus, it is urgent and important to develop an approach for identification of the sex of *B. papyrifera* at the seedling stage.

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Abbreviations: AFLP, Amplified fragment length polymorphism; **STS,** sequence tagged site.

Table 1. The sequence of amplified fragment length polymorphism (AFLP) and STS primers.

Primer name	Primer sequence (5'-3')
*EcoR*I-pre	gac tgc gta cca att ca
*Mse*I-pre	gat gag tcc tga gta ac
E-ACG	gac tgc gta cca att cac g
E-ACT	gac tgc gta cca att cac t
E-AGG	gac tgc gta cca att cag g
M-CTT	gat gag tcc tga gta act t
M-CAA	gat gag tcc tga gta a caa
M-CTG	gat gag tcc tga gta a ctg
MADB-1forward	tca gtt cca gtg acg acg ac
MADB-1reverse	cca act ttg aat ccg gaa aa
MADB-2forward	gaa ttc act caa gcc ctt tgg a
MADB-2reverse	tta act gta atc aag aac aaa gac tgg a

*EcoR*I-pre and *Mse*I-pre indicate the primers for pre-amplification. E-ACG, E-ACT and E-AGG reveal three different *EcoR*I-adapter selective amplification primers. M-CTT, M-CAA and M-CTG show diverse *Mse*I-adapter selective amplification primers. MADB-1 forward/MADB-1 reverse and MADB-2 forward /MADB-2 reverse are two pairs of STS primers.

Amplified fragment length polymorphism (AFLP), based on the selective PCR amplification technology was firstly developed by Vos et al. (1995). After the initial finding, this efficient, high-thought technology has been thought to be an important molecular marker, and was widely applied in a variety of organisms from bacteria and fungi to plants and animals (Bensch and Akesson, 2005; Hua et al., 2009; Meudt and Clarke, 2007). Consideration of laborious, time-consuming during its utilization for routine sex identification and mapping, AFLP markers were frequently converted into easy-operation approaches, that is, sequence tagged site (STS) and sequence-characterized amplified region (SCAR) markers (Hua et al., 2009). It reported that some AFLP-derived STS markers have been used for fine mapping of the sex gene in asparagus (Reamon-Büttner and Jung, 2000). Moreover, one of AFLP markers, termed as k2 fragment, was identified and converted into SCAR sequence, which could be used for marker-assisted breeding in *Brassica napus* (Ke et al., 2004). Since there are no available methods to identify the sex of *B. papyrifera* at the seedling stage, we consider developing a molecular approach to distinguish the sexes in *B. papyrifera*. In the present study, an AFLP molecular marker was screened, cloned and converted into STS sequence, which was introduced to the process of identification of gene sex in *B. papyrifera* during seedling stage.

MATERIALS AND METHODS

Collection of plant

Young leaves of male and female plants of *B. papyrifera* were harvested based on the presence of male flowers or development of fruits from National Baiwang Forest Park, Beijing, China.

DNA extraction and AFLP analysis

Total genomic DNA was extracted separately from 3 g fresh leaves from four male and four female individuals with the modified sodium dodecyl sulphate (SDS) standard method (Danilova and Karlov ., 2006), and its quality and quantity were analyzed using 1% agarose gel electrophoresis and an ND-1000 spectrophotometer (NanoDrop Technologies, USA).

AFLP analysis was conducted as described by Ma et al. (2010) with minor modifications. In brief, 100 ng of genomic DNA was digested with 1 µl mixture of *EcoR*I and *Mse*I (1.25 µ/µl , TAKARA, Japan) for 7 h at 37°C, then heated for 15 min at 70°C to inactivate the enzymes. Ligation of specific adapters to restriction fragments was performed by adding 12 µl adapter mixture and incubated for 12 h at 20°C, and then the ligation reaction products was diluted for 10 times. Pre-amplification PCR was performed in a 50 µl volume with 2 µl 10×EasyTaq buffer, 4 µl 2.5 mM dNTPs, 0.5 µl EasyTaq DNA polymerase, 2 µl *EcoR*I-pre primer (Table 1), 2 µl *Mse*I-pre primer (Table 1) and 3 µl diluted ligation mixture; sterilized water was added to make a final volume of 50 µl. The PCR reaction involved an initial 5 min denaturation at 94°C; followed by 20 cycles of 94°C, 30 s; 56°C, 1 min; 72°C, 1 min; and a final 7 min of extension. Aliquots of individual PCR products were separated on 1% agarose gel and stained with ethidium bromide. The pre-amplified PCR products were diluted to 30 times.

Nine primer combinations were used for selective amplification (Table 1). The PCR amplification was performed using a 'touchdown' program: one cycle of denaturation at 94°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 1 min; 12 cycles of subsequently lowering the annealing temperature (65°C) by 0.7℃ per cycle while keeping at 94°C for 30 s (denaturation) and 72°C for 1 min (extension); 25 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. The PCR products were separated by 6% denaturing polyacrylamide gel.

Cloning and sequence analysis of male-specific AFLP marker

The fragment of interest was excised with a razor blade from a 6% denaturing polyacrylamide gel and redissolved in 50 µl of Tris-EDTA buffer (TE, pH 8.0) at 100°C for 10 min. The re-amplification PCR reaction was performed with the corresponding primers and 5 µl of the TE buffer under the same conditions as before. PCR products were recovered from agarose gel, cloned into pMD-18 T vector and sequenced using ABI 3730xl automated DNA Sequencer (Invitrogen, China). The sequence was submitted to GenBank, and sequence homology was assayed using BLASTn methods at GenBank database (http://www.ncbi.nlm.nih.gov/blast).

Conversion of the male-specific AFLP marker to STS marker

Based on the sequence of the male-specific AFLP fragment, two pairs of STS primers termed as MADB-1 and MADB-2 (Male-Associated DNA from *B. papyrifera*), respectively were designed (Table 1) to convert the male-specific AFLP marker into the STS marker. To confirm the validity of these STS primers, they were used to amplify the male and female individuals of *B. papyrifera*. PCR was performed in a 50 µl volume with 2 µl 10×EasyTaq buffer, 4 µl 2.5 mM dNTPs, 0.5 µl EasyTaq DNA polymerase, 2 µl *EcoR*I selective amplification primer, 2 µl *Mse*I selective amplification primer, 3 µl diluted ligation mixture and distilled water was added to a final volume of 50 µl. PCR was conducted as follows: initial incubation at 95°C for 5 min, followed by 33 cycles of 95°C for 30 s, 60 to 66°C for 30 s; and 72°C for 60 s, with a final extension of 10 min at 72°C. The amplification was resolved on 1.5% agarose gel with a DL2000 DNA marker.

RESULTS

Screening, cloning and sequence analysis of male-specific AFLP marker

To identify male-specific DNA polymorphic fragments, nine primer combinations were used for AFLP analysis, which produced a total of 230 scorable bands with an average of 28.8 products per primer. One primer combination (*E*-ACT, *M*-CTG primer, Table 1) produced a significant fragment of 476 bp that was present in all male individuals but absent in all female samples (Figure 1).

The male-specific AFLP marker was amplified and recovered from the gels, cloned and sequenced (GenBank accession no. HQ202152). The sequence of the male-specific polymorphic AFLP marker is shown in Figure 2. BLAST results indicated that no homologous sequence was found in the GenBank database and complete open reading frame was also absent in this fragment.

Development of STS primer and sex identification via PCR in *B. papyrifera*

Since the long primers (19 to 25-mer) are more reliable and accurate than short AFLP primers (about 10-mer), we designed two pairs of STS primers, MADB-1 and MADB-2, based on the sequence of the male-specific AFLP fragments (Figure 2). To confirm their validity, these primers were used to amplify the male and female individuals. It was found that a 177 bp-long fragment was present in both male and female individuals using MADB-1 primers (Figure 3). It suggested that there exists considerable sequence homology between male and female samples. To identify the genetic sex of *B. papyrifera*, MADB-2 primers were used to test its suitability in identification of the plant sex. The result demonstrates that the appearance of fragment amplified via MADB-2 primer was closely related to annealing temperature. Although the fragment amplified from female samples was weak on agarose gel, the band appeared on both sexes of *B. papyrifera* with performance of relative low annealing temperature, 60, 62 and 64°C, (Figure 4a and b). However, the single 454 bp-length fragment existed in male *B. papyrifera* but none in females while the annealing temperature was increased to 66°C (Figure 4a and b). To further verify the reliability of this primer, another eight male and eight female samples were used to amplify the specific fragment with an annealing temperature of 66°C. The data were in accordance with the results described above (Figure 5).

DISCUSSION

Due to the different economic and medicinal values between male and female *B. papyrifera*, it’s an urgent need for early sex identification at the stage of seedlings on a large scale. Considering there are no available methods to distinguish the sexes before flowering. Efforts to identify dioecious plant sex type in an early stage of development are important for selecting female or hermaphrodite plants for transfer to the field, to gain time and reduce costs.

AFLP is a fast and simple technique which provides a large number of polymorphic markers without requiring any prior knowledge about the DNA sequences of the organisms. By now, this protocol has been used for identifying sex type of several dioecious plants such as *Asparagus officinalis* L. (Reamon-Büttner et al., 1998), *Rumex nivalis* (Stehlik and Blattner 2004), fig (Parrish et al., 2004) and Patagonian Pejerrey (Koshimizu et al., 2010). However, no available method has been devoted to discriminate the sexes in *B. papyrifera*. Therefore, we described an available approach to identify it, and the male-specific marker obtained from AFLP technique was proved to be reliable and efficient fragment.

It has been demonstrated that the majority of AFLP fragments were caused by single nucleotide polymorphisms (SNPs), insertion/deletion (indels) or point mutation at/within the restriction sites (Brugmans et al., 2003; Prins et al., 2001; von et al., 2003). The same length fragment appearance in both male and female samples indicated that the sex-linked marker was not caused by indel events. The PCR results from MADB-2 primers suggested that there were polymorphisms

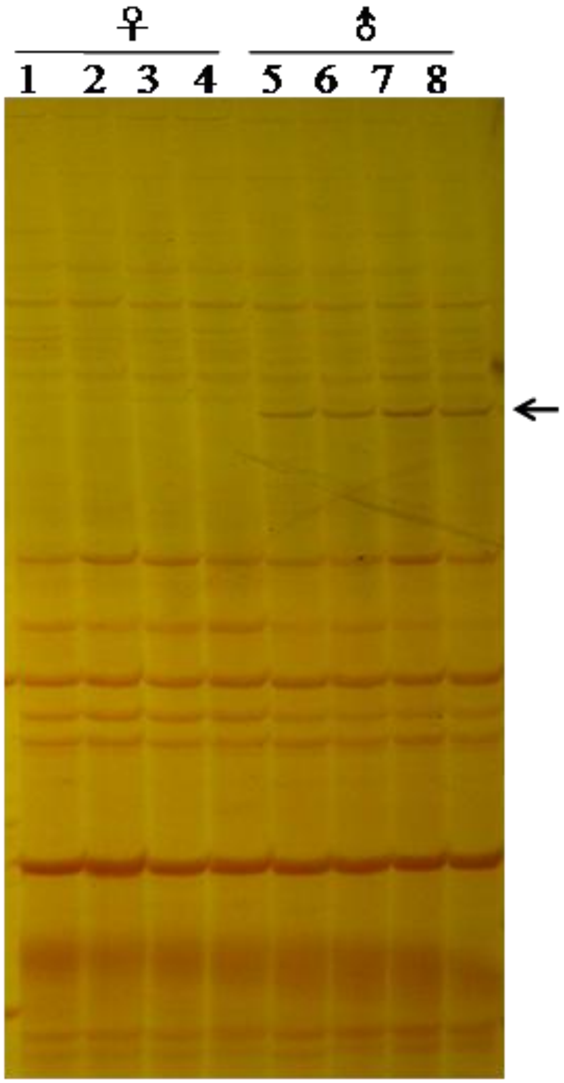

Figure 1. Male-specific AFLP marker obtained with E-AGG/M-CAA primer combination using 6% polyacrylamide denaturing gel. Lanes 1 to 4 female (♀) individuals; lanes 5 to 8 male (♂) individuals. The male-specific AFLP marker is indicated by arrow.

Primer MADB-2 forward

GACTGCGTACCAATTCACTCAAGCCCTTTGGATCGCGACTTAGAAGCAAATCTCCGAA
GCCCCAGTTCTTCGCCAATAGCTCTTCGCTGAGTGATCTCTGAACTCTTAGGGAGTTAC
AAGTGTTGGACCGATTTGGGCAGCCGGGAACTCCGTCATCAAGGCCCGTAACCGTGGA
TCTAAAAAGCCGTTCAATAACGACGACGACGACTTCTATGGCTACAAGGATTCCGACG
GCAGCGATATCGACTATGATA*tcagttccagtgacgacgac*GATGATGACGTTGACATGGCTTTTG
ATGACGACGACGAGGAGGACGTTGGCAAGAAAGGGAAGAAGAGAAAGTAAAAGAATT
TGAATATCATCATCGTTATTTGACCATTGTTTTGGGAAATGTTATTTGGTCTCTG*ttttccggat*
*tcaaagttgg*TCTTGTCCAGTCTTTGTTCTTGATTACAG**TTACTCAGGACTCATC**

Primer MADB-2 reverse

Figure 2. DNA sequence of *B. papyrifera* male-specific AFLP-STS marker. Sequences in bold, E-AGG and M-CAA primers; sequences in small italic character, MADB-1 STS primers; sequences with underline, MADB-2 STS primers.

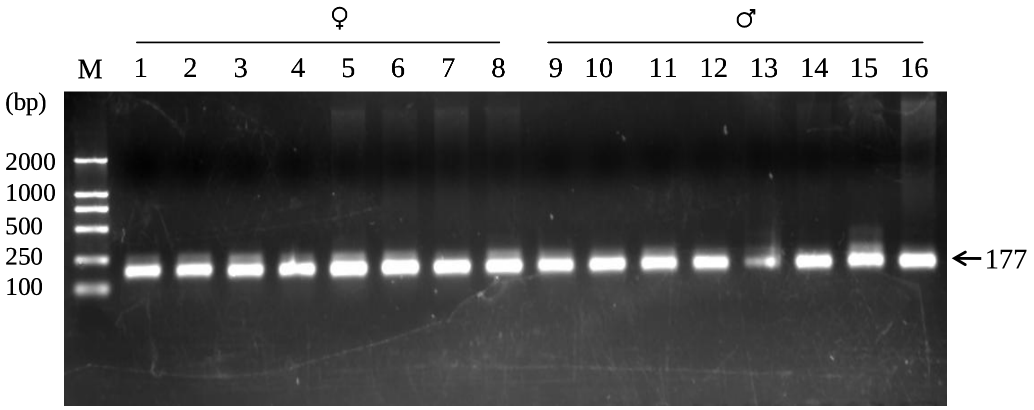

Figure 3. The amplification of *B. papyrifera* using MADB-1 STS primers with annealing temperature of 60°C. M, Marker; Lanes 1 to 8, female (♀) individuals; lanes 9 to 16 male (♂) individuals.

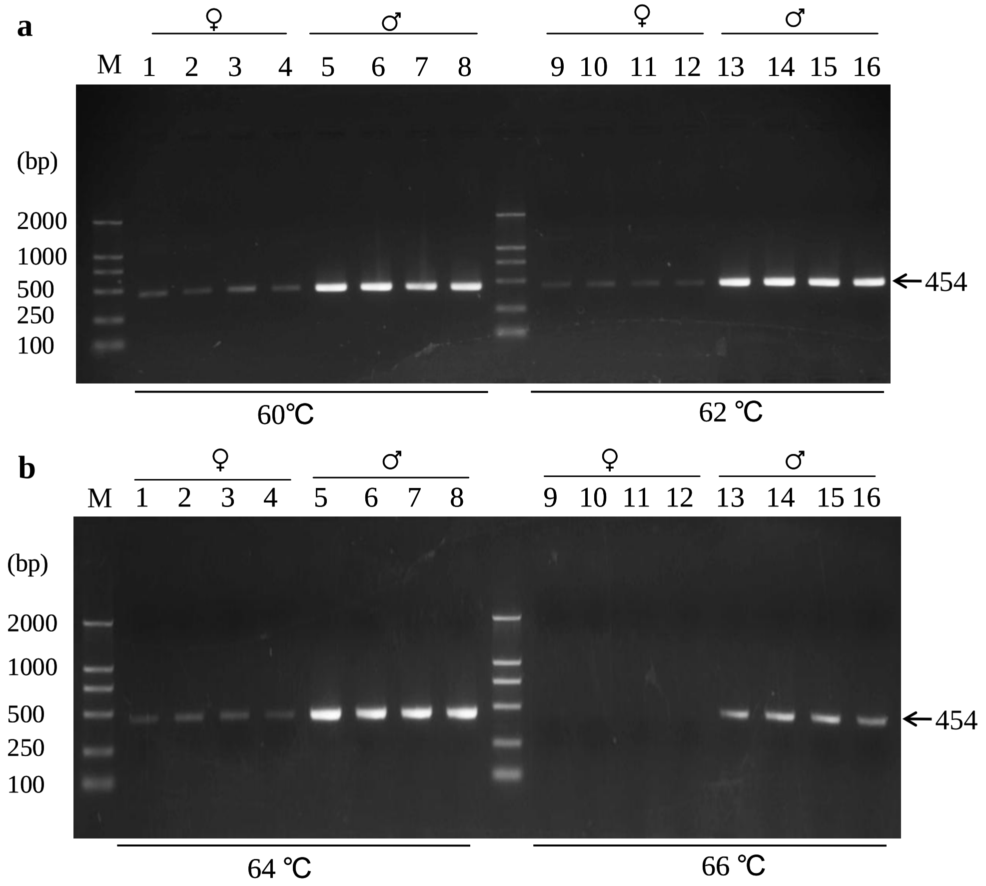

Figure 4. Determination of optimum annealing temperature using MADB-2 STS primers. M, Marker; Lanes a1 to a4, a9 to a12, b1 to b4 and b9 to b12, female (♀) individuals; Lanes a5 to a8, a13 to a16, b5 to b8 and b13 to b16, male (♂) individuals. Lanes a1 to a8, Annealing temperature of 60°C; Lanes a9 to a16, annealing temperature of 62°C; Lanes b1 to b8, annealing temperature of 64°C; Lanes b9 to b16, annealing temperature of 66°C.

between male and female plants, and this difference was significantly affected by annealing temperature. We assume that point mutants at or within the restriction sites might lead to the sequence polymorphisms of the partial

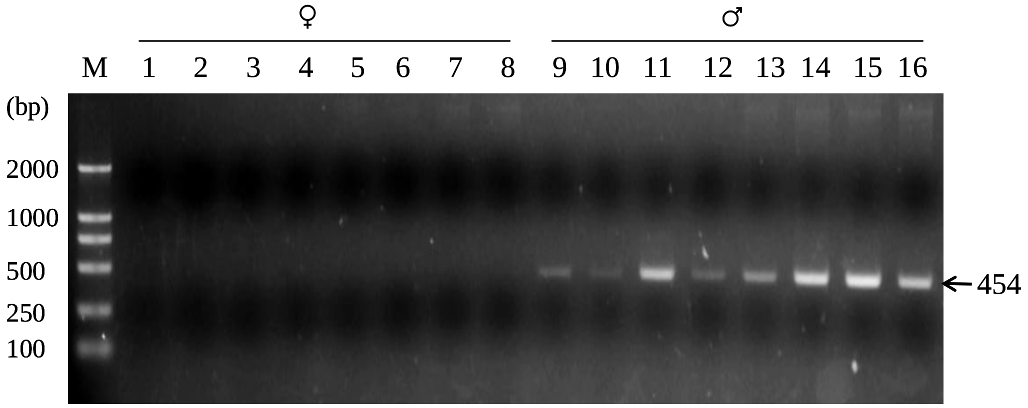

Figure 5. The verification of STS marker using another 16 individuals of *B. papyrifera* and MADB-2 STS primers with annealing temperature of 66°C. M, Marker, Lanes 1 to 8, female (♀) individuals; Lanes 9 to16 male (♂) individuals.

genome in *B. papyrifera*.

In conclusion, we have provided an efficient and reliable AFLP-dependent molecular technique to identify the sex type of *B. papyrifera* based on STS marker derived from AFLP sequence. This marker can be used for large-scale screening of sex type of *B. papyrifera* at the stage of seedlings.

ACKNOWLEDGEMENTS

This work was supported by The National Project for Public Industry of China (No. nyhyzx07-012).

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