# DEVELOPMENT OF SEX SPECIFIC MOLECULAR MARKERS IN DIOECIOUS *PIPER LONGUM.L* PLANTS BY DIFFERENTIAL DISPLAY

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**ABSTRACT**

In the absence of any genetic or sequence information of a closely related species or individuals, showing morphological similarity and limited difference, differential display becomes an important technique to look into their genetic similarity and differences. Plants showing difference like disease resistance and susceptibility can be screened by this technique and sequence homology search can be carried out. Once some potent cDNA fragments were identified, they can be sequenced for full length gene and also design specific primers to screen the particular trait. Using this technique we could identify male and female plants of *Piper longum* at a younger stage.

**Key words:** *Differential display, Dioecious Piper longum. L, sex determination, SCAR marker, reverse northern*

## 1. INTRODUCTION

*Piper longum* L., a dioecious plant species, is well known for the medicinal value of its different plant parts For example, mature female spikes are highly valued for their use in the treatment of upper respiratory tract diseases in traditional and ayurvedic medicine in India [1]. In the medicinally important plant species, *Piperlongum.L*, family Piperaceae commonly known as Pippali,

Male and female plants differ in the morphology of their spikes, which bear minute achlamydous unisexual flowers. Mature female spikes, known, as 'long Pepper' is shorter and thicker than the male spikes. *Piper longum* plants are functionally dioecious and have no distinguishing cytological or vegetative features to identify the sex of plants. Both male (having long spikes) and female plants (having short spikes), which grow in the wild under humid and shady conditions, remain indistinguishable until the flowering stage and maintain their sexual identity through out their successive generations. In populations where flowering selections are desired, the requirement to grow plants to maturity before eliminating the unwanted males represents a considerable loss in plant maintenance. Minute achlamydous flowers are closely packed in the fleshy axis of the spikes. Since seeds are very minute and not available, the plant is vegetatively propagated through stem cuttings and through tillers arising from the base of mature plants. The plant maintains its sexual identity through out based on its origin. A method for identifying the sex of plants at younger stage would therefore help in elimination of unwanted plants [2].

Differential display is PCR based method, which is a very sensitive technique. It is a fingerprinting technology that facilitates the identification of mRNAs in a cell or tissue, can be used to compare mRNA levels in many samples. Thousands of genes embedded in the genome of an organism are selectively expressed in different tissues or organs. Any abnormality in expression causes a variety of pathological conditions or disease states. The isolation and characterization of differentially expressed genes becomes the first step to understand such pathological conditions. Even weakly expressed PCR products, partially representing the transcripts are viewed in the polyacrylamide gel under denaturing conditions. Once the differential display bands are identified, it is eluted and reamplified. These differential, partial cDNA fragments are cloned into plasmid vector. Then their differential expression is confirmed and sequenced [3]. Using these clones DNA as probe, we can screen cDNA library and full-length cDNA of male and female plants of *Piperlongum* can be cloned and sequenced by standard procedure.

The RAPD technique [4, 5] has been used to develop DNA markers linked to sexual phenotype in *P. longum* [2], *Silene latifolia* [6], *Pistacia vera* [7], *Salix viminalis* [8], *Myristica fragrans* (nutmeg), *Cannabis sativa* [9] and *Actinidia* species [10]. It has been suggested that the RAPD markers should be converted to sequence characterized amplified region (SCAR) markers based on their DNA sequence, which could be detected through polymerase chain reaction (PCR) with longer sequence-specific primers [11]. Therefore, several of the sex-linked random amplified polymorphic DNA (RAPD) markers have been converted into SCAR markers i.e., In *Salix viminalis* [12] in *Actinidia chinensis* (10) in Papaya [13] and several resistance RAPD markers converted to SCAR [11, 14]. Male-specific SCAR markers developed on the basis of respective RAPD marker sequences in *Humulus lupulus* [15] *Asparagus officialis*, *Actinidia chinensis* [10] and *Cannabis sativus* [9]; one in each species and five in *Silene latifolia* [6]. Gill *et al*., [10] developed one female sex-specific SCAR marker in *Asparagus* by following a similar strategy.

## 2. MATERIALS AND METHODS

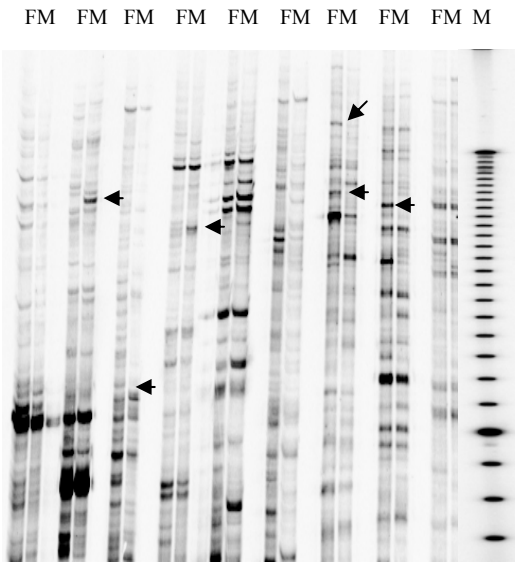
### 2.1 RNA preparation and reverse transcription

Genes that are differentially expressed between male and female plant spikes of *Piperlongum* was analyzed by isolating RNA from the developing spikes and screening the mRNA, by the PCR based method. Using TRIZOL reagent (Gibco BRL) RNA was isolated. Total RNA was treated with Dnase1 (10U) to remove any traces of genomic DNA present. Each RNA sample was subjected to three separate reverse-transcription reactions, each with one of the three one-base-anchored oligo-dT primers (2µm) and arbitrary primers through PCR. RT is done in three PCR tubes (3 for male and 3 for female), each contain one of the 3 different one base anchored oligodT primers $HT_{11}$ G (5′-AAGCTTTTTTTTTTG-3′), $HT_{11}$A (5′-AAGCTTTTTTTTTTA-3′), $HT_{11}$C (5′-AAGCTTTTTTTTTTC-3′), 2µm each along with 250µm dNTPs and 100 units of MMLV reverse transcriptase. PCR is done at 65°C for 5 minutes, 37°C for 60 minutes and 72°C for 5 minutes. Differential display analysis was performed on the reverse-transcribed products using the corresponding oligo-dT primers and arbitrary 13-mer primers (2µm). The 20µl reaction medium contained 50 ng of reverse-transcribed product, 1X PCR buffer, 25 µM dNTPs, 2µM arbitrary primer, 2 µM anchor primer, 0.2 µl of γ [$^{33}$P] labeled dATP (2000Ci/nmol) and 5 units of Taq DNA polymerase (Promega). The polymerase chain reaction (PCR) condition were as follows: 94°C for 30 s; 40°C for 2 min; 72°C 30 s; for 40 cycles followed by 72°C for 5 min [17] in a thermocycler (Eppendorf) and electrophoresed it in a sequencing gel for high resolution of amplified cDNA [16].

The reamplified products added with formamide dye (98% formamide, 10mM EDTA, 0.1% Bromophenol blue, 0.1% Xylene cyanol) were run in 6% denaturing polyacrylamide gel (PAGE) (Urea 7.5m, 20:1 Acry:Bis acry) in 1XTBE buffer, at 45 W, 1700 volts, for 2.5 h, dried one hour at 80°C without fixing. After seeing autoradiogram of 72 hours exposure, differentially seen bands were located (Fig A), cutout, and excised gel pirces were rehydrated in 100 µl of sterile water for 10 min. The swollen gel pieces were boiled for 15 min and centrifuged at 10 000 g for 2 min. The DNA was precipitated from the supernatant using 3M Sodium acetate, glycogen (100 µg/ml), and 2 volume ethanol for 30 min at 70°C. The precipitate was rinsed with 85 % ethanol and dissolved in 10 µl sterile water. Repeatability is checked; eluted fragments were reamplified following the same PCR conditions used for amplification of the reverse-transcribed product, but with non-radioactive dNTPs only. From the 18 primer combinations of anchored (A, G & T) oligo

dT primers and arbitrary primers (AP1, AP2, AP3, AP4, AP7 & AP8) used, 48 differential fragments were obtained (32 from female plant and 16 from male plant).

*Figure 1. Differential display using one-base anchored oligo-dT primers ($AAGCT_{11}A$, $AAGCT_{11}G$ and $AAGCT_{11}C$) in combinations with 3 arbitrary 13-mer primers AP1, AP2 & AP3.*




Arrow indicates the bands showing difference were eluted. M-RNA samples from male(M) *Piperlongu*m plants. F- RNA samples from female (F)*Piperlongu*m plants. M-Molecular weight marker.

### 2.2 Reverse northern screening

Reverse northern analysis was carried out on the differentially expressed cDNA fragments. Prominently reamplified cDNA fragments (13 from female and 5 from male) ranging from 150 bp to 250 bp were dot blotted. 1µgm each of the PCR product (reamplified cDNA fragments) were blotted to a nylon membrane in duplicate, air dried for 30 minutes, denatured for 5 minutes at room temperature by placing on (DNA side up) Whatman paper presoaked with denaturing solution (0.5 mNaOH, 1.5 mNaCl) washed thrice in neutralizing solution (0.5 MNaOH, 1.5 MNaCl, 1 mMEDTA) for 5 min each at room temperature, rinsed in 2XSSC (300 mM Sodium citrate, 3 M NaCl). Membrane was UV cross linked (biorad) and prehybridised (7% SDS, 10 mMEDTA, 0.5 MNa2HPO4, pH 7.2) for 2 hours.

For reverse northern hybridization, cDNA probes were synthesized from 1µg of DNA-free RNA, each of male and female plants, incubated with oligodT12-18 (1µg) at 70°C for 10 minutes, placed in ice for 1 minute, 1X PCR buffer, 1.5mM Mgcl2, DTT (200mM), d (GAT)TP(20 mM) mix were added and radiolabeled with 75µci (3000 ci/nmol) α [$^{32}$P] dCTP using MMLVRT(100U) at 42°C, for 50 min. For reverse northern hybridization, the blots were wetted with 2X SSC and pre-hybridized (0.5 M $Na_2HPO_4$ [pH 7.2], 2 mm EDTA, 7% SDS, 1% BSA and 10 µg/ml denatured salmon sperm DNA) at 65 °C for 4h. A denatured probe was added to the pre-hybridization reaction and hybridization was continued for 16 h at 65 °C.

Reverse Northern hybridization (10) was done with both the blots using female (Fig.2A) and male (Fig.2B) RNA probes respectively. After reverse northern, blot was washed twice with 50 ml 2X SSC and 0.1% SDS for 15 min each at 65°C. The hybridized membrane was exposed to a phosphor screen and the exposed screen was scanned using Phosphor Imager (BioRad) and analysis was performed using Quantity One software (BioRad). Among the dot blot positive DNA, 8 were PCR product originated from female plant (A1, B1, C1, C2, C3, C6 & A2) and 3 were originated from male plant (B4, B5 & B6).

*Figure 2. Dot blot (in duplicate), reverse northern hybridization.*

Figure 2A

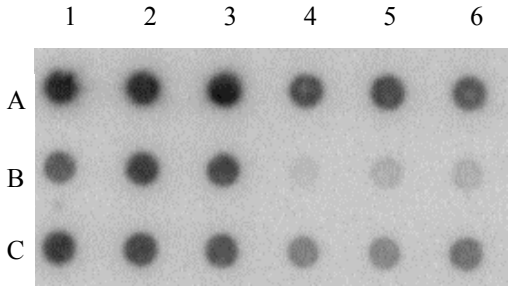
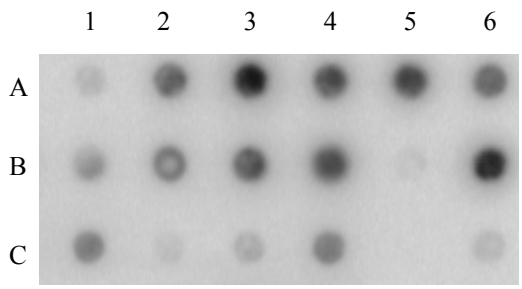


Figure 2B




Reverse Northern hybridization using female (Fig. 2A) and male (Fig. 2B) plant RNA as probe. A1 to A6, B1 to B6 and C1 to C6 were the eluted and reamplified fragments from Poly-acrylamide gel shown in Fig.1.

### 2.3 Cloning and sequencing of the differential bands

Reverse northern [18] positive fragments were cloned using 150ng PCR-TRAP Vector (Genehunter Corporation cat.no.P404). Out of 200 Tetracycline (20µg/ml) resistant colonies obtained form the cloning of all the above PCR products, few (35clones) were checked for insert [19] with primers (lgh & rgh) specific to vector. Sizes obtained were 200 bp to 350 bp (≈ +120bp) including the regions of the vector (Fig.3A, 3B, 3C). The clones were sequenced using an ABI 3730 automated DNA sequencer (Applied Biosystems) using the ABI BigDye terminator cycle sequencing ready reaction kit, version 3.0 and all the sequences were submitted to databases at the National Centre for Biotechnology Information (NCBI) as ESTs (AY 168429 to 168439).

Primers developed based on the flanking sequences of the above ESTs were used to screen the male and female *Piper longum* genomic DNA. Primers were developed as per the following. FADPA1-Forward and FADPA1-Reverse primer developed from Gen bank sequence AY168429, FADPD6-Forward and FADPD6-Reverse primer from Gen bank sequence AY168431, FADPC6-Forward and FADPC6-Reverse primer from Gen bank sequence AY168430, FADPE19-Forward and FADPE19-Reverse primer from Genbank sequence AY168432, MADPA1-Forward and MADPA1-Reverse primer from Genbank sequence AY168437, FADPA33-Forward and FADPA33-Reverse primer from Genbank sequence AY168433, FADPD118-Forward and FADPD118-Reverse primer from Genbank sequence AY168436, MADP8-Forward and MADP8-Reverse primer from Genbank sequence AY168439, FADPC-Forward and FADPC-Reverse primer from Genbank sequence AY168435, FADPB-Forward and FADPB-Reverse primer from Genbank sequence AY168434 and MADP5-Forward and MADP5-Reverse primer from GenBank AY168438, sequences (Table. 1).Based on the PCR product obtained from the genomic DNA of male and female *Piper longum* plants we were able to easily differentiate male and female plants at their younger stage (Fig.3). The sequences were named MAD (Male associated DNA from nomenclature proposed by Sakamoto *et al*., [20], FAD (Female associated DNA) and primers as MADP and FADP mRNA samples analyzed side by side allows differentially expressed genes to be identified and recovered can be used as probes and for cloning their cDNA or genomic DNA and also for designing specific primers. mRNA differential display method has several technical advantages over subtractive hybridization. This is based on PCR and DNA sequencing gel electrophoresis. Five microgram of total RNA is sufficient to cover all the anchored oligo-dT primers used in all combinations of arbitrary 13 mers. Reverse northern is quick and easy way to verify induced mRNAs identified by differential display or subtractive hybridization and mRNAs with abundance level as low as 1 in 40,000 can be detected easily [21].

With our knowledge, this may be the first report on development of sex specific markers from differentially displayed products for the identification of sex in dioecious plants.

Acknowledgements

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*Table. 1. Table showing the sequences of the primers developed from differentially displayed fragment sequences. TM- Annealing temperature, bp- base pair, FADP- female associated DNA primer, MADP- male associated DNA primer (Nomenclature proposed by Sakamoto et al. 1995). F- Forward, R- Reverse.*

| Genbank Accn.no. | Primer sequence | Primer ID | TM | size |
|---|---|---|---|---|
| 1. AY168429 | 5'TTTGTATAATCAATAATCTGTGG3'<br>5'AAGCTTTGGTCCGGGGAGTCC3' | FADPA1-F<br>FADPA1-R | 62°C | 232bp |
| 2. AY168431 | 5'CAAAAAGAGTGTATATATTTA3'<br>5'AAGCTTTGGTCAGGTTATA3' | FADPD6-F<br>FADPD6-R | 51°C | 129bp |
| 3. AY168430 | 5'GAGGCAAACTGTTCTCTTTTTA3'<br>5'AAGCTTTGGTCAGTGAATGGT3' | FADPC6-F<br>FADPC6-R | 60°C | 136bp |
| 4. AY168432 | 5'GGTCAGGAGGAATGTTGCGTT3'<br>5'ATTTCATCGAAAAAAAAAGCTT3' | FADPE19-F<br>FADPE19-R | 59°C | 142bp |
| 5. AY168437 | 5'ACAAGATGAAATTGAACAATA3'<br>5'GGTATTAGAGGGTGTCCATTA3' | MADPA1-F<br>MADPA1-R | 56°C | 203bp |
| 6. AY168433 | 5'CAGATGGAAGCTTTCGGCTCTT3'<br>5'CCCACTTGCTGCATATTGAGG3' | FADPA33-F<br>FADPA33-R | 62°C | 214bp |
| 7. AY168436 | 5'GTCAGGTAGACGGCAGAGTAC3'<br>5'ACACAAATATAGTCCAACACTA3' | FADPD-F<br>FADPD-R | 62°C | 118bp |
| 8. AY168439 | 5'CTTTGAGATCACTTCTCATTA3'<br>5'ACTCATAACCATTACATTCAC3' | MADP8-F<br>MADP8-R | 56°C | 134bp |
| 9. AY168435 | 5'GGTCAGGGGAAGGTAGGCCTT3'<br>5'TAAAGTATGGTAATTGATAGT3' | FADPC-F<br>FADPC-R | 54°C | 139bp |
| 10. AY168434 | 5'CAGTCTCAGCTGACGTCGATA3'<br>5'GAATGAAACCACCATGAACTTG3' | FADPB-F<br>FADPB-R | 62°C | 187bp |
| 11. AY168438 | 5'ATTTCTAGGCACCATTGATGG3'<br>5'AAGTTTACTCTTTGAACTTGA3' | MADP5-F<br>MADP5-R | 56°C | 210bp |

*Figure 3. Agarose gel showing the amplified fragments from the genomic DNA of male and female Piper longum plants using the primers designed from differentially displayed fragment sequences (Table. 1).*

Figure 3A

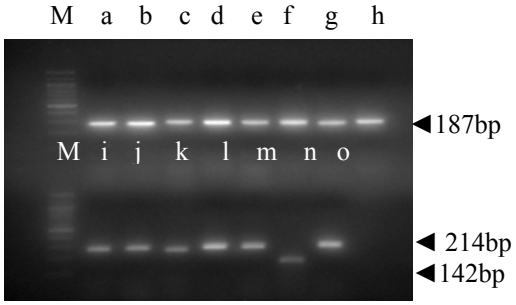


Figure 3A. Fragments 187bp from female plants(a to h) amplified with primer set 10, 214bp fragments (I to m, o) amplified from female plants using primer set 6, 142bp (h) fragment amplified from female plants using primer set 4.

Figure 3B

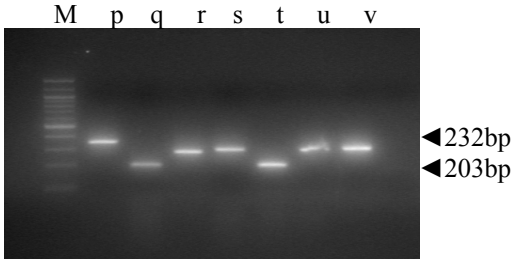


Figure 3B. Product of 232bp(r, s, u, v) amplified from female plants using primer set 1, 203bp product (q, t) amplified from male plants using primer set 5.

Figure 3C

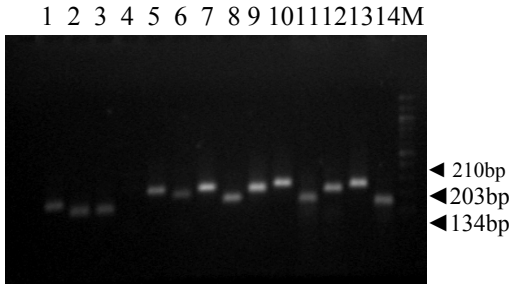


Fig 3C. Product of 210 bp (7, 9, 12) amplified from male plants using primer set 11, 203bp products (5, 6, 8, 11, 14) amplified from male plants using primer set 5, 134 bp product (1, 2, 3) amplified from male plants using primer set 8. M- Molecular weight marker.

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