HAYATI Journal of Biosciences, March 2009, p 21-24 Vol. 16, No. 1
ISSN: 1978-3019

Chloroplast DNA Copy Number May Link to Sex Determination in *Leucadendron* (Proteaceae)

MADE PHARMAWATI[1]*, GUIJUN YAN[2], PATRICK FINNEGAN[2]

[1]Biology Department, Faculty of Mathematics and Natural Sciences, Udayana University, Kampus Bukit Jimbaran, Bali 80361, Indonesia

[2]School of Plant Biology, Faculty of Natural and Agricultural Sciences, The University of Western Australia, 35 Stirling Highway, Crawley, 6009, WA, Australia

Received October 21, 2008/Accepted March 27, 2009

***Leucadendron* (Proteaceae) is a South African genus, the flowers of which have become a popular item in the Australian cut-flower industry. All species are dioecious. In general the female flowers are the more desirable as cut flowers. The availability of a molecular marker linked to sex determination is therefore needed both to maximize the efficiency of breeding programs and to supply markets with flowers from the preferred sex. The polymerase chain reaction-based method of suppression subtractive hybridization (SSH) combined with mirror orientation selection (MOS) were applied in an attempt to identify genome differences between male and female plants of *Leucadendron discolor*. Screening of 416 clones from a male-subtracted genomic DNA library and 282 clones from a female-subtracted library identified 13 candidates for male-specific genomic fragments. Sequence analyses of the 13 candidate DNA fragments showed that they were fragments of the chloroplast DNA, raising the possibility that chloroplast DNA copy number is linked to sex determination in *Leucadendron*.**

Key words: *Leucadendron*, sex determination, suppression subtractive hybridization (SSH)

INTRODUCTION

All *Leucadendron* species are known to be dioecious. The *Leucadendron* Breeding Program UWA has produced both male and female hybrids. However except for *L. discolor* female flowers are generally preferred, due to the long picking period and the ease of handling during processing. Therefore a larger female seedling population is needed to obtain female selections. Some important agricultural crops such as papaya, kiwifruit and asparagus are dioecious and the sex of most dioecious plants can be determined only at flowering time. A number of dioecious plants such as *Silene latifolia* (Lengerova *et al*. 2003) and *Humulus lupulus* (Karlov *et al*. 2003) show distinct sex chromosomes. The sex of these plants can be determined based on cytology. However, most dioecious plants do not show morphologically different sex chromosomes, examples include *Actinidia deliciosa* (Ferguson *et al*. 1997) and *Atriplex garrettii* (Ruas *et al*. 1998). The breeding program would become more efficient if methods for early identification of female and male plants were available.

Various molecular methods have been widely used to identify sex-specific DNA fragments. The random amplified polymorphic DNA (RAPD) technique has been used successfully to identify male-specific DNA fragment in *Carica papaya* (Urasaki *et al*. 2002). Restriction fragment length polymorphisms (RFLP) and amplified fragment length polymorphism (AFLP) have been used to diagnose sex in *Asparagus officinalis* (Biffi *et al*. 1995; Reamon-Büttner *et al*. 1998).

*Corresponding author. Phone: +62-361-701954 ext 235, Fax: +62-361-703137, E-mail: pharmawati@hotmail.com

In this study suppression subtractive hybridisation (SSH) combined with mirror orientation selection (MOS) of genomic DNA, were used in an attempt to identify genome differences between male and female *L. discolor*.

MATERIALS AND METHODS

Driver and Tester Preparation. Genomic DNA was isolated from five female and five male *L. discolor* plants using DNeasy plant mini kit (Qiagen, Clifton Hill, VIC, Australia). As much as 0.1 g leaves were ground to a fine powder in liquid nitrogen with a mortar and pestle. DNA was isolated according to manufacturer's instruction. Driver and tester preparations were conducted based on Diatchenko *et al*. (1996). In this study two sets of subtractions were conducted. Male DNA was driver in one substraction and female DNA was driver in the other.

SSH + MOS and Cloning of Cubstructed DNA. Suppression subtractive hybridisation combined with mirror orientation selection (SSH + MOS) was done according to Rebrikov *et al.* (2000). PCR products from SSH + MOS experiment were purified (Perfectprep Gel Cleanup Kit, Eppendorf) and inserted into pGEM-T vector (Promega) according to manufacturer's instructions. Detection of clones containing insert was done by colour selection of colonies according to Sambrook *et al.* (1989).

Differential Screening of Subtracted *L. discolor* DNA Library. Inserted MOS products were amplified using NP_2Rs as a primer in 25 ìl reaction mixtures containing 1 x PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3; Eppendorf), 2.5 ìM of primer, 2 mM $MgCl_2$, 1 U of *Taq* polymerase (Eppendorf), 0.2 mM of each dNTP (Promega). The amplification program

was one cycle of initial denaturation at 95 °C for 3 min, 25 cycles at 94 °C for 30 sec, 68 °C for 30 sec, 72 °C for 1.5 min followed by one cycle at 72 °C for 7 min. Amplified inserts were arrayed on positively charged nylon membranes to perform dotblotting analysis. The membranes were screened by hybridisation with *Rsa*I digested genomic DNA from male and female *L. discolor* labelled by DIG (Roche, Castle Hill, NSW, Australia) according to manufacturer's instruction.

DNA Sequencing. Nucleotide sequences of plasmid inserts were determined using T_7 and/or SP_6 primers by the dideoxynucleotide chain termination method (BigDye v3.1, ABI, Western Australia). The products were separated and analysed by The West Australian Genome Resource Centre. The characterization of sequencing data was done using the BLAST program at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). To test the similarity of insert sequences, the sequences were aligned using ClustalW (Thompson *et al.* 1994 and BioManager by ANGIS: http://www.angis.org.au).

RESULTS

Suppression subtractive hybridisation and mirror orientation selection was done in two directions, with male as tester in one set and as driver in other. MOS after male subtraction produced a similar PCR pattern to MOS after female subtraction except there was a very faint band present in MOS after male subtraction. To identify the fragments amplified by MOS, two areas were cut from the gel; one area where a faint band was present in male but it was not present in female and another area with a smear PCR product (Figure 1). Those selected PCR products were purified, ligated into pGEM-T vector and transformed into *Escherichia coli*.

As many as 470 clones from the male subtracted MOS library and 320 clones from the female-subtracted library were picked. Among the MOS clones, 416 male subtracted clones (88.5%) and 282 female subtracted clones (88.1%) contained inserts (data not shown). The amplified inserts were dot blotted onto nylon membranes and hybridised with DIG-labeled *Rsa*I digested male and female genomic DNA to assay for differential representation. The 282 inserts from the female subtracted MOS library showed similar signal when hybridised to male and female *Rsa*I digested genomic DNA (Figure 2), with no female specific clone detected. Of the 416 male subtracted MOS clones, 13 had differentially presented DNA, suggesting male specificity (Figure 3, Table 1).

Sequencing of 13 inserts which had differentially presented DNA was conducted and BLAST searches identified 12 inserts as chloroplast DNA fragments and one insert was a DNA fragment that encoded polyprotein from *Oryza australiensis* (Table 1). Analysis of the insert sequences showed that clones 354 and 356 were identical.

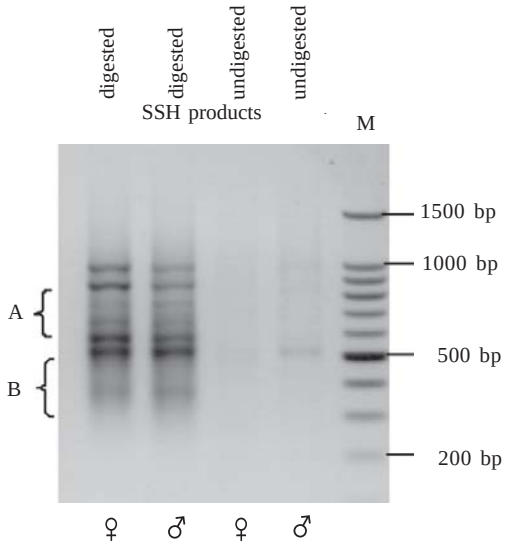

Figure 1. Analysis of MOS PCR amplification. M: is the 100 bp ladder. The PCR products were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide. Sizes of marker bands shown on the right. A and B were two areas that were predicted to have genome differencies between male and female *Leucadendron*. These areas were cut and cloned.

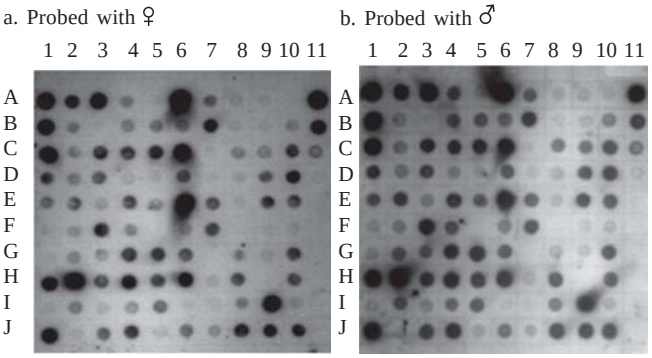

Figure 2. Differential screening of female MOS subtracted genomic DNA library showing signal similarities between female MOS subtracted genomic DNA library hybridised with probe made from *Rsa*I digested female (a) and male (b) genomic DNA of *L. discolor* using DIG labeling. One hundred clones from a total of 282 clones are shown. Dots in column 11 of each blot are positive controls of genomic DNA (rows A to D) in serial dilution. Negative controls of lambda DNA and bluescript plasmid are in the same row in column E and F.

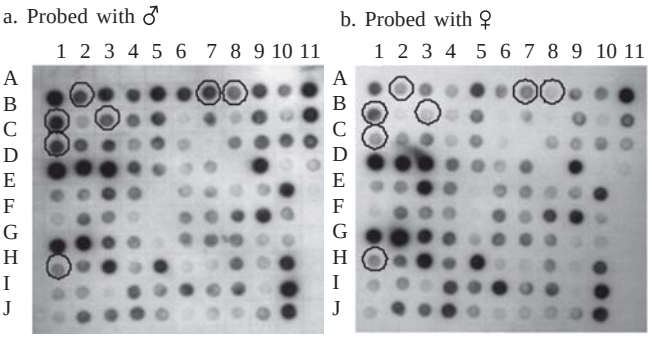

Figure 3. Differential screening of male MOS subtracted genomic DNA library showing inserts with different signal intensities (circled dots) between male MOS subtracted genomic DNA library probed with *Rsa*I digested male (a) and female (b) genomic DNA of *L. discolor* using DIG labeling. One hundred clones from a total of 416 clones are shown. Dots in column 11 of each blot are positive controls of genomic DNA (rows A to D) in serial dilution. Negative controls of lambda DNA and bluescript plasmid are in the same row in column E and F.

Table 1. Characteristics of the 13 clones of SSH-MOS products which showed differently presented DNA

Insert number	Approx. size (bp)*	Clear readable sequence (bp)	Accession number	Gene(s)	Organism	Sequence identity	E-value
2	600	220	AJ627251	cpDNA 23S rRNA	*Nymphaea alba*	182/188 (96%)	1e-88
3	710	725	AY586361	cpDNA NADH dehydrogenase subunit F	*Fagus grandifolia*	466/477 (97%)	0.0
11	500	272	AJ428413	cpDNA *rpo*C2	*Calycanthus fertilis* var. ferax	213/229 (93%)	4e-86
17	470	463	Z00044554304	cpDNA *rrn*23	*Nicotiana tabacum*	429/438 (97%	0.0
30	450	252	AY582139	cpDNA *psa*B	*Panax ginseng*	228/239 (95%)	7e-103
101	1000	321	U63020	cpDNA D1 protein (*psb*A)	*Magnolia pyramidata*	284/309 (91%)	1e-108
197	620	617	AJ428413	cpDNA *pet*B and *pet*D	*Calycanthus fertilis* var. ferax	543/593 (91%)	0.0
204**	510	321	BAA22288	polyprotein (mat_peptides)	*Oryza australiensis*	88/106 (83%)	2e-37
220	680	680	AJ428413	cpDNA *ycf*6-*psb*M intergenic spacer	*Calycanthus fertilis* var. ferax	104/121 (85%) 62/70 (88%) 55/66 (83%)	2e-19 2e-10
240	510	503	AJ970307	cpDNA16S rRNA and tRNA Ile	*Cucumis sativus*	466/477 (97%)	0.0
354	620	639	AJ428413	cpDNA 23S rRNA and tRNA Ala	*Calycanthus fertilis* var. ferax	600/609 (98%)	0.0
356	620	639	AJ428413	cpDNA 23S rRNA and tRNA Ala	*Calycanthus fertilis* var. ferax	601/612 (98%)	0.0
362	650	662	AJ428413	cpDNA tRNA Ile and tRNA Ala	*Calycanthus fertilis* var. ferax	336/347(96%)	5e-168

*Determine by gel electrophoresis of amplified insert using NP_2Rs primer; **BLASTX was used to identify homology because BLASTN resulted in low identity.

DISCUSSION

The results indicate that there was similarity of hybridisation signal intensities among the DNA fragments obtained when they were probed with *Rsa*I-digested genomic DNA. This implies that the male and female genomes of *L. discolor* showed homology. Hybridisation of female-MOS subtracted DNA to probes prepared from *Rsa*I-digested male genomic DNA and female genomic DNA showed signal similarities. In contrast, in the hybridisation of male-MOS subtracted DNA, no clone hybridised only with male genomic DNA, but 13 inserts (Table 1) repeatedly produced stronger signals when probed with *Rsa*I-digested male genomic DNA than with *Rsa*I digested female genomic DNA. Differences in hybridisation efficiencies to both probes indicate that the clones/inserts may represent genes or DNA fragments with different number of copies per genome (Rebrikov *et al*. 2000). The lack of sex specificity of sex-fragments was observed in other dioecious plant (Ruas *et al*. 1998) where sex-specific DNA fragments hybridised to genomic DNA from both sexes.

Thirteen clones of male subtraction which produced more intense signal were sequenced. Comparison of the sequences against the sequences from genebank database (BLAST search) showed that 12 clones matched with chloroplast DNA and one clone matched with polyprotein (mat_peptides) from *Oryza australiensis*. This suggests that the copy number of chloroplast DNA per unit mass of leaf may be higher in male than female *L. discolor*.

In the human Y-chromosome, genes could be classified into two classes. The first class contains housekeeping genes which express in many organs, while the second class consists of Y-chromosomal gene families which specifically expressed in testis (Lahn *et al*. 2001). Compared to those of sex-specific genes in human, the Y-linked genes in *S. latifolia* are members of the first class genes which are not likely to be involved in sex determination but belong to a group of housekeeping genes (Filatov 2005). For examples the *SlY4* and *SlX4* as well as the *DD44Y* and *DD44X* from *S. latifolia*, respectively encode the oligomycin sensitivity-conferring protein (OSCP), an essential component of the mitochondrial ATP synthase (Moore *et al*. 2003), and fructose-2,6-biphosphatases that are probably involved in carbohydrate metabolism (Atanassov *et al*. 2001).

The finding that chloroplast DNA presents in different copy number in male and female *L. discolor* may link to sex determination. Recent study found that there was an accumulation of chloroplast DNA on the Y-chromosome of *S. latifolia* (Kejnovsky *et al*. 2006). The finding that the chloroplast copy number was higher in male *L. discolor* than in female *L. discolor* suggests that chloroplast DNA of *L. discolor* may be integrated into certain chromosomes.

Although genes encoded OSCP and fructose-2,6-biphosphatases in *S. latifolia* are classified as housekeeping genes, they may influence sex. Mutations of mtDNA which affect the function of genes encoding subunit of the mitochondrial ATP synthase will reduced ATP production and can lead to cytoplasmic male sterility (CMS) (reviewed by Mackenzie & McIntosh 1999). Like mitochondria, chloroplasts are energy-generating organelles. Therefore, it is suggestive that chloroplasts may have a role in determining sex.

The finding that in humans, the activity of the steroidogenic acute regulatory protein (StAR), a mitochondrial protein required for fetal sexual differentiation, is regulated by the rate of mitochondrial protein import (Bose *et al*. 2002) showed that mitochondria are involved in sex determination. In mammals males have higher metabolic rate than females, hence, it has been hypothesized that there is a difference in the number and activity of mitochondria in developing male

and female mammals and that divergence in energy metabolism may be a basic difference between sexes (Mittwoch 2004). Thus, a similar hypothesis can be made for chloroplast.

SSH + MOS showed that in *Leucadendron* differentially represented DNA is very low in abundance. In papaya, it was observed that the non-recombining region of sex chromosomes is only 10% of the whole chromosome (Liu *et al*. 2004). It is predicted that the non-recombining region in sex chromosomes of *Leucadendron* may also be very small, making the identification of sex-linked genes more difficult.

ACKNOWLEDGEMENT

M.P. thanks AUSAID for providing a PhD scholarship, Ralph Sedgley from Amarilo Protea for providing plant material, Ben Croxford (UWA) for assistance in plant material collection, David Thirkettle-Watts for technical assistance in molecular biology.

REFFERENCES

Atanassov I *et al*. 2001. Analysis and evolution of two functional Y-linked loci in a plant sex chromosome system. *Mol Biol Evol* 18:2162-2168.

Biffi R *et al*. 1995. A restriction fragment length polymorphism probe for early diagnosis of gender in *Asparagus officinalis*. *Hort Sci* 30:1463-1464.

Bose HS, Lingappa VR, Miller WL. 2002. Rapid regulation of steroidogenesis by mitochondrial protein import. *Nature* 417:87-91.

Diatchenko L *et al*. 1996. Suppression subtractive hybridisation: A. method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc Natl Acad Sci USA* 93:6025-6030.

Ferguson AR, O'Brien IEW, Yan GJ. 1997. Ploidy in Actinidia. *Acta Hort* 444:67-71.

Filatov DA. 2005. Substitution rates in a new *Silene latifolia* sex-linked gene, *SlssX/Y. Mol Biol Evol* 22:402-408.

Karlov GI, Danilova TV, Horlemann C, Weber G. 2003. Molecular cytogenetics in hop *(Humulus lupulus* L.) and identification of sex chromosomes by DAPI-banding. *Euphytica* 132:185-190.

Kejnovsky E *et al*. 2006. Accumulation of chloroplast DNA sequences on the Y chromosome of *Silene latifolia*. *Genetica* 128:167-175.

Lahn BT, Pearson NM, Jegalian K. 2001 The human Y chromosome, in the light of evolution. *Nat Genet* 2:207-216.

Lengerova M, Moore RC, Grant SR, Vyskot B. 2003. The sex chromosomes of *Silene latifolia* revisited and revised. *Genetics* 165:935-938.

Liu Z *et al*. 2004. A primitive Y chromosome in papaya marks incipient sex chromosome evolution. *Nature* 427:348-352.

Mackenzie S, McIntosh L. 1999. Higher plant mitochondria. *Plant Cell* 11:571-585.

Mittwoch U. 2004. The elusive action of sex-determining genes: mitochondria to the rescue. *J Theor Biol* 228:359-365.

Moore RC *et al*. 2003. Genetic and functional analysis of *DD44*, a sex-linked gene from the dioecious plant *Silene latifolia*, provides clues to early events in sex chromosome evolution. *Genetics* 163:321-334.

Reamon-Büttner SM, Schondelmaier J, Jung C. 1998. AFLP markers tightly linked to the sex locus in *Asparagus officinalis*. *Mol Breeding* 4:91-98.

Rebrikov DV *et al*. 2000. Mirror orientation selection (MOS): a method for eliminating false positive clones from libraries generated by uppression subtractive hybridisation. *Nucl Acid Res* 28:1-4.

Ruas CF *et al*. 1998. Male-specific DNA in the dioecious species *Atriplex garrettii* (Chenopodiaceae). *Am J Bot* 85:162-167.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning. A laboratory manual*. USA: Cold Spring Harbor, Laboratory Pr.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl Acid Res* 22:4673-4680.

Urasaki N *et al*. 2002. A male and hermaphrodite specific RAPD marker for papaya (*Carica papaya* L.). *Theor Appl Genet* 104:281-285.