Research Article

Isolation and DNA-Marker Based Genotyping of Individual Pollen Grains

A. Naseer Aziz

Department of Agricultural and Environmental Science, College of Agriculture, Human and Natural Sciences,
Tennessee State University, 3500 John A. Merritt Boulevard, Nashville, TN 37013, USA.

***Corresponding author: E-mail: aaziz@tnstate.edu,**

Phone # (615) 963-1595, fax # (615) 963-1557

ABSTRACT

Dry pollen of diploid *Solanum* clone 9507-04, Cotton chromosomal substitution line and *Echinacea purpurea* 'Magnus' were isolated and their cells contents released in individual tubes containing water or pollen germination media, respectively. Six polymorphic markers from individual pollen grains of the diploid potato were amplified using two random amplified polymorphic DNA (RAPD) primers, i.e., UBC291 and UBC504. The procedures for primer extension pre-amplification (PEP) for genome and subsequent amplified fragment length polymorphism (AFLP) analysis with 13 primers pairs were tested on the upland cotton line. The cotton parental AFLP markers were scored for their presence or absences in the 35 pollen samples and majority of the markers were found monomorphic as expected from the gametes of a near isogenic line. PEP and AFLP procedures for linkage mapping were standardized using purple cone flower pollen grains. From 64 possible AFLP primer pairs, 37.5% amplified 104 markers for cone flower that were linked in 11 linkage groups ranging from 13 to 134 centimorgans (cM). The 409 cM sized linkage map constructed for *E. purpurea* 'Magnus' genome had average distance of 2.4 cM to 22 cM for the two to 55 polymorphic markers identified within the 11 groups. The protocols described in this report would serve to enhance breeding efficiencies in plants important for food, fiber, ornamental and medicinal industries.

KeyWords: Coneflower; Diploid potato; Isolated pollen; Individual pollen grains; Molecular Marker; AFLP; RAPD; PEP; Linkage mapping; Pima cotton; Upland cotton; Chromosomal substitution line.

Introduction

Molecular markers' based plant evaluation for cultivar release is more efficient in terms of time and development costs than conventional breeding approaches [1]. The segregation of parental markers, during anthesis, into its pollen grains can be used by plant breeders for genetic analyses. Plant breeding efficiencies can be enhanced if such pollen based segregation of molecular markers is used in genetic linkage estimations [2]. Pollen is a convenient source of DNA as they require only limited freezer storage space and are easily stored for long periods [3, 4]. DNA typing of individual pollen grains has several uses including assessment of gamete merit. Pollen have been subjected to selection processes since pollen characteristics such as resistance to different forms of stresses have been used to preselect pollen for fertilization in subsequent genetic hybridizations [5]. In the development of polyploid varieties unreduced (2n) pollen, that can be produced by first or second division restitution [6, 7], are used. Unreduced pollen produced by nondisjunction at first meiotic division would retain the heterozygous loci of the parent, whereas nondisjunction at the second meiotic division would produce the 2n pollen

segregating for dominant and recessive loci of the parent [8]. Therefore, the segregation analysis of molecular markers in unreduced pollen would help to understand the origin of 2n pollen production. Amplified fragment length polymorphism (AFLP) analysis of rose showed that they produce unreduced male and female gametes [9]. Pollen protoplasts also contain several organelles which contribute to markers' amplification products [4, 10], therefore such molecular markers could also be used to study the inheritance of organelles in the pollen populations. AFLP markers have been used to identify male fertility restorer locus [11]. Molecular markers' based analysis of individual gametes can be used to compute genetic linkage relationships by determining whether the individual gametes are parental or recombinant in genotype [2, 12]. Generation of AFLPs' based data from individual pollen grains for genetic mapping is efficient and cost approach since it can circumvent the need to maintain large plant populations while conducting controlled pollinations [13, 14].

Two approaches have been reported to extract DNA from isolated pollengrains. One approach would be to capture single pollen grain in a PCR tube after

drilling a hole in the wall of a pollen grain with a UV-laser microbeam [14]. Alternatively a pollen grain isolated from dry spread was dispersed in liquid to release its cell content [4]. Mature pollen grains contain three haploid nuclei, i.e., one vegetative nucleus and two sperm nuclei [10]. Although individual pollen grains contain very limited amounts of DNA, polymerase chain reaction (PCR) techniques can be used to amplify DNA from genomic (chromosomal) sequences [4]. Since PCR is capable of amplifying a DNA sequence on the order of 10^9 fold [12], PCR-based molecular markers in an individual cell can be analysed. For single gamete genotyping, random amplified polymorphic DNA (RAPDs) and AFLPs are more useful than other PCR-based markers since these can be used without any prior genome information and analysed with less effort [15]. In RAPD and AFLP analyses, the dominant markers are identified by the presence of DNA bands and recessive markers are identified by the absence of the DNA bands [16]. Despite dominance of the markers, RAPD and AFLP can provide the resolution needed with less effort [15]. Use of the gametophytic tissue in RAPD and AFLP analysis can facilitate the detection of co-dominant markers [17] which are otherwise rarely discernible [16]. Since a heterozygous genotype (for an allelic pair) segregates into gametes carrying dominant and recessive markers [17], analysis of pollen via RAPD and AFLP markers would simplify the genetic assessment of the parent since only 1:1 segregation of the markers is expected [2, 13].

AFLP based markers are more reliable than RAPDs, since it is based on restriction digest and stringent PCR conditions [1]. However, restriction digest, which is needed for AFLP analysis, is not feasible with the limited amount of DNA available in single cells. Though single cell gametes have sufficient DNA for PCR amplification [18], the small amount of DNA in a pollen grain needs to be increased for subsequent AFLP analysis. The primer extension pre-amplification (PEP) protocol of Zhang et al. [19] uses array of continuous random 15-mer primers to increase the amount of genomic DNA of a single cell sample. PEP-PCR has been since used for DNA amplification from animal [20], human [21] and plant [2] single cells. Improvements in PEP technique in order to maximize amplification of limited DNA samples have been made by combining more than one kind of DNA polymerase in different concentrations [22]. Gene Specific-Primer Extension Pre-amplification (GS-PEP) technique has been developed that amplifies gene of interest after increasing the copy number of genomic DNA [23]. Some PEP based sequence specific methods have been patented by combining randomized primers in

the first amplification reaction and specific primers in the second amplification reaction [24].

This study standardizes the individual pollen grain isolation and DNA harvest for subsequent PCR protocols using three unrelated genomes, i.e., diploid potato clone 9507-04, upland cotton line and purple coneflower plants. RAPD polymorphism from individual pollen grains of the diploid potato was also detected. The protocols for PEP amplification of genome and AFLP analysis were standardized using upland cotton line (with pima cotton's chromosome 17) and *Echinacea purpurea* 'Magnus' pollen grains. The procedures described in this report for isolation of individual pollen grain, release of pollen DNA for subsequent PCR amplification as well as molecular marker and genetic linkage analysis from individual pollen grains would serve as important tools for enhancing breeding efficiencies in plants.

Materials and Methods

Plant Materials and Growth

Diploid potato (*Solanum*) clone 9507-04 which had several species in its background (50% *tuberosum* and 50% wild species including *S. chacoense* and Argentinean wild potato species) were obtained and maintained in greenhouse as described [13]. *Echinacea purpurea* 'Magnus' starter plants from Green Leaf Perennials (Yoder Brothers Inc., Lancaster, PA, USA) were grown in controlled environment under flower inducing conditions [2]. Upland cotton chromosomal substitution (CS) line that has been created by deleting chromosome 17 and replacing it from Pima cotton was obtained in 2011 from Dr. Saha (Genetics and Precision Agriculture Research Unit, United States Department of Agriculture, Mississippi State, USA). The cotton aneuploid (*Gossypium hirsutum* x *G. barbadense*) plants had most of the genome from *G. hirsutum* TM-1 (Upland cotton) while substituting chromosomes number 17 from *G. barbadense* (B17) double haploid line 3-79 (Pima cotton) [25, 26]. These plants were grown in the greenhouse using standard nursery gallons (4.5 liters) that contained Miracle-Gro Potting Mix (Scott Co., Marysville, OH). Plants were maintained in a glasshouse at 24-28°C, watered as needed and fertilized bimonthly with 0.21-0.07-0.14 (N-P-K) (mgl-1) soluble fertilizer (Grace Horticultural Products Co., Cambridge, MA).

Leaf DNA Extraction

Bio 101 Fast Prep Tissue Disrupter System (speed 6 for 40 seconds) with bead mill tubes containing grinding matrix (Q. Biogene, Irvine, CA, USA) was used for 200 mg of plant tissue along with 1 ml of AP1 buffer solution (DNeasy® Plant Mini Kit (QIAGEN, Valencia, CA, USA). Leaf tissue was disrupted with Bio Fast Prep System set at

(Q. Biogene, Irvine, CA, USA). Four μl of RNaseA stock (100 mg/ml) solution was used to remove RNAs. Manufacturer's QIA-shredder spin column under centrifugation removed the debris. Precipitated DNA was then collected on DNeasy column membrane and eluted. DNA concentrations were quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) after verifying DNA isolation via 1% agarose gel (in TAE buffer) electrophoresis and using ethidium bromide ($0.5 \mu\text{gml}^{-1}$ in TAE buffer) stained gels that were visualized on UV trans-illuminator and photographed (AlphaImager 2000 system, Alpha Innotech, San Leandro, CA).

Pollen Harvest and DNA Release

Mature anthers containing dry pollen grains were shaken on a glass slide and placed under Nikon Diaphot TMD Biological Inverted Microscope (Nikon Instruments Inc., Melville, NY, USA). Austerlitz insect pin size 00 (thickness) and 37 mm long (Nasco, Fort Atkinson, WI, USA) mounted on Ultra-fine move Marzhauser (HS-6) micromanipulator (ALA Scientific Instruments, Inc., Westbury, NY, USA) was used to collect isolated pollen grains (Fig. 1). Single pollen grains were placed in PCR tubes (0.2 ml) containing 15 μl of the distilled water (potato) or 5 μl of pollen germination medium (coneflower and cotton).

Fig. 1: Isolation of individual pollen grain of (a) diploid *Solanum* clone 9507-04 and (b) *Echinacea purpurea* 'Magnus' on insect pin.

Fig. 2: Opening of (a) *Echinacea purpurea* 'Magnus' and (b) cotton pollen grain after incubation in respective germination media.

The germination medium for coneflower pollen consisted of 0.29 M sucrose, 1.62 mM H₃BO₃ and 1.27 mM Ca(NO₃)₂ and adjusted to pH 5.7 before autoclaving. The cotton pollen germination media contained 25% (w/v) sucrose, 0.52 mM KNO₃, 3.06 mM MnSO₄, 1.66 mM H₃BO₃, 0.42 mM MgSO₄ and 1 µM gibberellic acid, pH 7.6. The tip of insect pin was re-checked under the microscope prior to the next transfer to ensure pollen grains had been deposited into the liquid. Between each collection of the individual pollen the insect pin was cleaned consecutively with 70% and 100% ethyl-alcohol and dried. The isolated pollen grains in the individual PCR tubes were incubated for 1-2 hours at room temperature to release their DNA (Fig. 2). To avoid any possible enzymatic activity, all the samples were kept on ice and/or in a -20°C freezer after this incubation.

Genomic DNA Amplification

GENIUS Thermal Cycler (Techne Incorporated, Burlington, NJ, USA) was used for primer extension pre-amplification (PEP) of random genomic fragments with continuous random 15-mer poly nucleotide (SP180-2) primer (Operon technologies, Inc., Alameda, CA, USA). MasterAmp™ Extra-Long PCR kit (EPICENTRE®, Madison, WI, USA) along with PreMix-3 or PreMix-9 was used for PEP amplifications as described [2]. The PEP products were precipitated and collected on DNeasy mini spin column (QIAGEN, Valencia, CA, USA) before elution. DNA concentrations were quantified using a spectrophotometer (Eppendorf, Hamburg, Germany)

after verifying DNA amplification in ethidium bromide stained 1% agarose gel.

RAPD and AFLP Amplifications

PTC-100 Thermal Controller (MJ Research) used for RAPD amplifications with University of British Columbia (Vancouver, BC, Canada) primers UBC291 (5'-AGCTGAAGAG) and UBC504 (5'-ACCGTGCGTC).

For RAPD-PCR amplifications from *Solanum* clone 9507-04, single pollen grains (in 15 µl of water) or 10 ng DNA (from leaf) were used as DNA with 1 unit Ampli-Taq DNA polymerase (Perkin Elmer) while overlaid with a drop of fresh mineral oil. The amplification reactions were carried out using 45 cycles (default ramp time) of 1 min denaturation at 94°C, 1 min annealing at 36°C and 2 min extension at 72°C.

The GENIUS Thermal Cycler (Techne Incorporated, Burlington, NJ, USA) equipped with heating lid was used with AFLP System Analysis Kit (Invitrogen™ Life Technologies, Carlsbad, CA, USA). The adaptors and pre-selective primers (Table 1) as well as eight selective primers for *Mse*I adaptors (Table 2) were used for both coneflower and cotton samples as described [2, 27]. Agarose gel analyses were conducted to check restriction digestion, pre-amplification and amplification of the DNA samples from parental leaf DNA and pollen PEP products. Fluorescent Li-Cor (Inc., Lincoln, NE) IRDye® Phosphoramidite (IRD)-800 (optimized 800 nm infrared channels) labelled and dual (IRD 700 and 800) labeled selective primers for *Eco*RI adaptors were used for *Echinacea purpurea* 'Magnus' and for cotton CS-B17 analyses (Table 2) to generate AFLP markers, respectively.

Table 1. Nucleotide Sequences for amplified fragment length polymorphism adapter and no-selective (pre-) amplification primers designed for *Eco*RI and *Mse*I restriction cut sites.

	<i>Eco</i> RI	<i>Mse</i> I
Adaptors	5'-CTCGTAGACTGCGTACC CATCTGACGCATGGTTAA-5'	5'-GACGATGAGTCCTGAG TACTCAGGACTCAT-5'
Primers	5'-GACTGCGTACCAATTCA	5-GATGAGTCCTGAGTAAC

Table 2. Sequences of amplified fragment length polymorphism primers (provided by Invitrogen™ Life Technologies, Carlsbad, CA, USA) designed for *Eco*RI and *Mse*I adaptors.

IRD700/800* labeled primers for <i>Eco</i> RI adaptors	un-labeled primers for <i>Mse</i> I adaptors
5'-GACTGCGTACC AATTC AAC	5'-GATGAGTCCTGAG TAA CAA
5'-GACTGCGTACC AATTC AAG	5'-GATGAGTCCTGAG TAA CAC
5'-GACTGCGTACC AATTC ACA	5'-GATGAGTCCTGAG TAA CAG
5'-GACTGCGTACC AATTC ACT	5'-GATGAGTCCTGAG TAA CAT
5'-GACTGCGTACC AATTC ACC	5'-GATGAGTCCTGAG TAA CTA
5'-GACTGCGTACC AATTC ACG	5'-GATGAGTCCTGAG TAA CTC
5'-GACTGCGTACC AATTC AGC	5'-GATGAGTCCTGAG TAA CTG
5'-GACTGCGTACC AATTC AGG	5'-GATGAGTCCTGAG TAA CTT

* Labeling was with either or both fluorescent Li-Cor (Inc., Lincoln, NE) IRDye® Phosphoramidite (IRD)-700 and IRD-800 (optimized for Li-Cor 700 nm and 800 nm infrared channels) dyes for primers designed for *Eco*RI adaptors.

Gel Electrophoresis

For RAPD-PCR products' separation horizontal agarose gel electrophoresis system (Fisher Scientific, Pittsburgh, PA, USA) on 1.4% agarose gels (3.5Vcm⁻¹ for 3 hrs) stained with ethidium bromide (0.5µgml⁻¹) in 0.5X TBE (0.045M Tris, 0.045M Boric acid, 0.001 M EDTA) was used. Sizes of the ethidium bromide stained RAPD markers were determined by comparing with a DNA MassTM Ladder (InvitrogenTM Life Technologies, Carlsbad, CA, USA) after being visualized/ photographed over a UV transilluminator using an AlphaImager 2000 System (Alpha Innotech, San Leandro, CA, USA).

Before analysis on the DNA Sequencer, 3X blue loading dye (Li-Cor) was added to the AFLP samples. As described earlier [2, 27], the AFLP profiles from parent and from each pollen DNA sample (~0.6 µl) were and separated through electrophoresis using denature 6.5% polyacrylamide gel between 25cm² Soda-Lime (for single IRD-dye analyses) or Starphire® (for dual IRD-dyes analyses) glass plates that were 0.25mm apart. These DNA bands were scored, by simultaneously electrophoresing and analyzing the selective amplification products for approximately 3.5 hours, on Li-Cor's automated DNA analyzer (Global IR2 for coneflower or Global 4300 for cotton sample). The SagaTM Generation 2 Version 3.1 (Li-Cor) software was used for maintaining constant electrophoresis conditions of 1500V, 40W and 40mA at 45°C, and for scoring the presence or absence of parental AFLP markers in each pollen sample. Under Windows XP operating system (Microsoft Corporation, Redmond, WA, USA) the SagaTM software, lane and calibration modes was used to prepare gel for scoring.

Markers' and Linkage Analyses

Reproducible RAPD-PCR products from the potato parent, used as markers, were visually scored for their presence or absence in the pollen progeny. For each RAPD marker, goodness-of-fit to expected segregation of 1:1 (presence of DNA band: absence of DNA band) was tested by chi-square analysis using SYSTAT version 7.0 (SPSS Inc., Chicago, IL, USA). Segregation ratios that differed from the expected value (significant at P=0.05 or less) were classified as distorted [13].

Desmille lines (SagaTM software) were added to the gel pictures of cotton (IRD 700 and 800 based) and coneflower (only IRD-800 based) samples using monomorphic DNA-bands in the gel-lanes. Gels were then scored for the presence or absence of AFLP markers denoted by "+" and "-". Scored data were added to a database (Oracle® 9i) to generate reports consisting of segregation pattern (absence/presence in entire pollen progeny)

strings for each loci. Coneflower reports combined in a file were analyzed by genetic mapping software (JoinMap® 3.0, Plant Research International, Wageningen, Netherlands) for genetic linkage analysis and converting recombination fractions to map distances with Kosambi mapping function [2]. Linkage groups were thus constructed by using likelihood of odds (LOD) threshold of 3.0 and software's default maximal recombination fraction of 0.40 to determine the most likely marker orders inside groups. By lowering the LOD score to 2, unlinked loci were incorporated in linkage group one [2].

Results and Discussion

The segregation of parent plant's markers into its pollen grains during anthesis can be used for genetic analysis, which can be facilitated by single gamete genotyping. Using pollen of important food (potato) and nursery/medicinal (coneflower) crops this study standardizes the individual pollen grain isolation and DNA-fingerprinting protocols. Dry pollen from *Solanum* diploid clone 9507-04, cotton hypoauploid line CS-B17 and *Echinacea purpurea* 'Magnus' were successfully isolated individually. Observing under microscope a micro-manipulator was used to move an insect-pin (tip: ~0.1mm thickness) to touch individual pollen (Fig. 1) which would then adhere to the pin. Individual pollen grains were convenient to isolate, by adherence to tip of insect pin, from dry spread. Unlike too flexible hair-point [28], insect pin would move accurately with the help of micro-manipulator over the requisite location on microscope slide. Isolated single cells contents were released in individual tubes containing water or pollen germination media, respectively (Fig. 2). In agreement with Petersen *et al.* [4] and Krabe *et al.* [28] the pollen released their contents into water and germination media, respectively. Typically 60% pollen thus isolated would succeed in PCR amplification. A few nonspecific and low intensity PCR products would also be discernible, however, it is unlikely that such extraneous DNA is in quantitative competition [29] with pollen DNA or causes significant errors in genotyping.

Six polymorphic markers from individual pollen grains of the diploid potato were amplified using two random amplified polymorphic DNA (RAPD) primers, i.e., UBC291 and UBC504. Some RAPD markers showed expected ratio of 1:1 segregation of 'presence of band: absence of band' while others showed distorted ratio. No linkage analysis was conducted for potato pollen grains since only six polymorphic RAPD markers were amplified. Since only those pollen samples that were successful for amplification of the PEP products as well as the parental AFLPs were used for subsequent marker

analysis, some distorted segregation was expected. The procedures for primer extension pre-amplification (PEP) for genome and subsequent amplified fragment length polymorphism (AFLP) analysis with 13 primers pairs (Table 3) were tested on the upland cotton line [30]. Many genomic copies in fragments of varied lengths were produced through PEP procedure by amplifying the tri-nucleate DNAs of coneflower and cotton pollen grains [2, 30]. The MasterAmp™ Long PCR kit (EPICENTRE®, Madison, WI) was used since its enhancers increased the fidelity and accuracy of the resulting PEP products [31]. Thus larger sized (>300 base pairs) and increased number of AFLP markers were yielded when pollen PEP amplifications were conducted along with MasterAmp™ Long PCR kit. The parental AFLPs of cotton were marked as present or absent in the 35 pollen PEP products and majority of the markers were found monomorphic [30]. No linkage estimations were needed for cotton pollen grains

since majority of the AFLP markers amplified from those samples were found monomorphic, as expected from the gametes of a near isogenic chromosomal substitution line [25, 26]. PEP and AFLP procedures for linkage mapping were standardized using germinated purple coneflower pollen. Table 4 lists all the primer pairs that were identified for AFLP amplifications for *E. purpurea* 'Magnus' genome. The 104 parental coneflower AFLPs were placed in 409 centimorgans (cM) linkage map after pollen analyses [2]. The resulting 11 linkage groups ranging from 13 cM to 134 cM in sizes had two to 55 polymorphic markers each with average mapping distance of 2.4 cM to 22 cM. The protocols described in this report provide an opportunity to directly observe the meiotic division products. Such innovative genetic analyses tools would serve to enhance breeding efficiencies in plants important for food, fiber, ornamental and medicinal industries.

Table 3. Amplified fragment length polymorphism (AFLP) primer pairs producing polymorphic markers for genetic analysis in *G. hirsutum* CS-B17 line.

AFLP* Primer pairs	Labelling Dye ¹	No. of Polymorphic AFLPs (size in kD)
1) E-AGG/M-CAA	IRDye® 800	9 (306, 210, 199, 160, 151, 143, 93, 84, 65)
2) E-ACC/M-CTT	IRDye® 800	8 (243, 186, 150, 123, 107, 91, 76, 63)
3) E-ACC/M-CTA	IRDye® 700	7 (356, 251, 236, 181, 173, 121, 78)
4) E-ACC/M-CTG	IRDye® 700	9 (319, 259, 190, 166, 160, 115, 87, 64, 54)
5) E-ACC/M-CAT	IRDye® 700	6 (292, 277, 133, 125, 115, 107)
6) E-ACA/M-CTC	IRDye® 700	4 (159, 140, 106, 77)
7) E-ACA/M-CTT	IRDye® 700	6 (265, 237, 203, 174, 173, 137)
8) E-ACC/M-CAG	IRDye® 700	8 (152, 148, 142, 135, 89, 85, 80, 75)
9) E-ACC/M-CAG	IRDye® 800	13 (210, 192, 166, 152, 148, 142, 135, 112, 101, 97, 89, 80, 68)
10) E-AGC/M-CAT	IRDye® 800	5 (199, 129, 124, 119, 62)
11) E-ACC/M-CTG	IRDye® 800	5 (297, 259, 244, 190, 54)
12) E-ACC/M-CTC	IRDye® 800	7 (179, 163, 138, 131, 124, 87, 76)
13) E-AGG/M-CTG	IRDye® 800	only monomorphic bands amplified

*Each AFLP (amplified fragment length polymorphism) marker is identified by the primer-pair designed for both *EcoRI* (E) and *MseI* (M) adaptors, and the three selective nucleotides of each primer

¹Primers designed for *EcoRI* adaptors had been labelled by Li-Cor (Inc., Lincoln, NE) IRDye® Phosphoramidite fluorescent dyes optimized for Li-Cor 700 nm (IRD-700) or 800 nm (IRD-800) infrared channels.

Table 4. Amplified fragment length polymorphism primer pairs producing polymorphic markers for genetic analysis in *Echinacea purpurea* 'Magnus'.

E-ACA/M-CAA, E-ACC/M-CAA, E-ACA/M-CAC, E-ACC/M-CAC, E-ACG/M-CAC, E-ACT/M-CAC, E-ACG/M-CAG, E-ACT/M-CAG, E-AGC/M-CAG, E-AGG/M-CAG, E-AAG/M-CAT, E-ACC/M-CAT, E-ACT/M-CAT, E-AGG/M-CAT, E-AGC/M-CTA, E-ACA/M-CTC, E-ACT/M-CTC, E-ACA/M-CTG, E-ACC/M-CTG, E-ACT/M-CTG, E-AGG/M-CTG, E-ACA/M-CTT, E-ACG/M-CTT, E-AGG/M-CTT.

*Each pair consists of primers designed for both *EcoRI* (E) and *MseI* (M) adaptors. The three selective nucleotides of each primer are shown.

Acknowledgments

Author would like to thank Ms. S. Bhatti, Mr. A. Gardner, Mr. S. Allaby, Dr. R. Sauve, Mr. M. Jennings, Ms. A. Sherrell, Mr. T. Tillman and Ms. C. Gibson and Ms. D. Long for facilitating the maintenance of the experimental plants as well as Ms. M. Stevens, Ms. L. Douglas, Ms. V. Burns, Ms. C. Robinson, Ms. V. Kankarla, Mr. R. Jackson and Ms. L. S. Kommireddy and Mr. R. Harrison for technical assistance. This work was supported by Tennessee State University, United States Department of Agriculture, University of New Brunswick and Agri-Food Canada.

References

- Vos P., Zabeau M., Hogers R., Bleeker M., Reijans M., van de Lee T., Hornes M., Frijters A., Pot J., Peleman J., and Kuiper M. (1995) AFLP: A new technique for DNA fingerprinting. *Nucl Acids Res.* **23**:4407-4414.
- Aziz A.N. and Sauve R.J. (2008). Genetic Mapping of *Echinacea purpurea* via Individual Pollen DNA Fingerprinting. *Mol Breeding*, **21**: 227-232.
- Dubouzet J.G., Hong C.J. and Etoh T. (1996) Comparison of random amplified polymorphic DNA profiles of crude extracts of pollen DNA in *Allium*, using *Thermus aquaticus* and *Pyrrococcus furiosus* DNA polymerase combinations. *Mem Fac Agr Kagoshima Univ.* **32**:43-49.
- Petersen G., Johansen B. and Seberg O. (1996) PCR and sequencing from a single pollen grain. *Plant Mol Biol.* **31**:189-91.
- Orenstein J., Nachmias A., Colon L.T. and Hoogendorn J. (1994). The effect of *Verticillium dahliae* phytotoxin on germination of potato pollen. *Isr J Plant Sci.* **42**:29-36.
- Ortiz R. and Peloquin S.J. (1994) Use of 24 chromosome potatoes (diploids and dihaploids) for genetic analysis. In: J. E. Bradshaw and G. R. Mackay. *Potato genetics. Centre for Agriculture and Bioscience, International, Cambridge, UK*. pp. 133-154.
- Tai G.C.C. (1994) Use of 2n gametes. In: J. E. Bradshaw and G. R. Mackay. *Potato genetics. Centre for Agriculture and Bioscience, International, Cambridge, UK*. pp. 109-32.
- Cappadocia M., Cheng D.S.K. and Simonette R.L. (1984) Plant regeneration from in vitro culture of anthers of *Solanum chacoense* Bitt. and interspecific diploid hybrids *S. tuberosum* L. x *S. chacoense* Bitt. *Theor Appl Genet.* **69**:139-43.
- Crespel L. and Gudin S. (2003) Evidence for the production of unreduced gametes by tetraploid *Rosa hybrida* L. *Euphytica*. **133**: 65-69.
- Desprez B., Chupeau Y. and Bourgin J.P. (1995). Preparation and fusion properties of protoplasts from mature pollen of *Nicotiana glauca*. *Plant Cell Rep.* **14**:199-203.
- Touzet P., Hueber N., Burkholz A., Barnes S. and Cuguen J. (2004) Genetic analysis of male fertility restoration in wild cytoplasmic male sterility G of beet. *Theor. Appl Genet.* **109**: 240-247.
- Arnheim N. (1989) A new approach to constructing genetic maps: PCR analysis of DNA sequences in individual gametes. In: H. A. Erlich. *PCR technology: Principles and applications for DNA amplification. M Stockton Press, New York, USA*. pp. 119-135.
- Aziz A.N., Seabrook J.E.A. and Tai G.C.C. (1999) Amplification of RAPD markers from individual pollen grains of a diploid *Solanum* clone 9507-04. *Am Potato J.* **76**:179-182.
- Matsunaga S., Schütze K., Donnison I.S., Grant S.R., Kuroiwa T. and Kawano S. (1999) Single pollen typing combined with laser-mediated manipulation. *The Plant Journal*. **20**:371-378.
- de Bruin A., Ibelings, B.W. and Van Donk E. (2003) Molecular techniques in phytoplankton research: from allozyme electrophoresis to genomics. *Hydrobiologia*. **491**: 47-63.
- Williams J.G.K., Kubelik A.R., Livak K.J., Rafalski J.A. and Tingey S.V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucl Acids Res.* **18**: 6531-6535.
- Horn P. and Rafalski A. (1992). Nondestructive RAPD genetic diagnostics of microspore derived *Brassica* embryos. *Plant Mol Biol Rep.* **10**:285-93.
- Leefflang E.P., McPeck M. S. and Arnheim N. (1996) Analysis of meiotic segregation, using single sperm typing: Meiotic derive at the myotonic dystrophy locus. *Am J Hum Genet.* **59**:896-904.
- Zhang L., Cui X., Sciimitt K., Hubert R. and Navidi W. (1992) Whole genome amplification from single cell: Implications for genetic analysis. *Proc Natl Acad Sci, USA.* **89**:5847-5851.
- Tominaga K. and Hamada Y. (2004) Efficient production of sex-identified and cryosurvived bovine in-vitro produced blastocysts. *Theriogenology.* **61**: 1181-1191.
- Von-Eggeling F., Michel S., Guenther M., Schimmel B. and Claussen U. (1997). Determination of the origin of single nucleated cells in maternal circulation by means of random PCR and a set of length polymorphisms. *Human-Genetics.* **99**: 266-270.
- Casas E. and Kirkpatrick B.W. (1996). Evaluation of different amplification protocols for use in primer-extension preamplification. *Biotechniques.* **20**: 219-225
- Yoshizumi M., Kohno T., Nishioka M., Sawada T.

- and Yokota J.(2002)Gene Specific-Primer Extension Pre amplification (GS-PEP), a simple method to increase the copy number of a gene prior to PCR amplification. *Proc Japan Acad Series B PhysBiol Sci.* **78B**: 123-128.
24. Dietmaier W. and Ruschoff J. (2002)Method of primer-extension preamplification PCR.*Official-Gazette-of-the-United-States-Patent-and-Trademark-Office-Patents.1257(1).* US 6365375 UPDATE CODE: 20020430.
25. Saha S., Wu J., Jenkins J.N., McCarty Jr. J.C., Gutierrez O.A., Stelly D.M., Percy R.G. and Raska D.A. (2004) Effect of Chromosome Substitutions from *Gossypiumbarbadense*L. 3-79 into *G. hirsutum*L. TM-1 on Agronomic and Fiber Traits. *J Cotton Sci.***8**:162-169.
26. Stelly D.M., Saha S., Raska D.A., Jenkins J.N., McCarty J.C. and Gutierrez A.O.(2005) Registration of 14 Upland (*Gossypiumhirsutum*) germplasm lines disomic for different *G. barbadense* chromosome or arm substitutions. *Crop Sci.***45**: 2663-2665.
27. Aziz A.N., Sauve R.J. and Tegegne F.(2007). Cost of AFLP-Based-Fingerprinting for Phylo-Genetic and Genetic-Linkage Analyses for the Purple Coneflower, Several St. John's Wort and Goldenseal Accessions. *ActaHorticulturae (International Society for Horticultural Science).***756**: 85-94.
- 28.Krabel D., Vornam B. and Herzog S.(1998) PCR-based random amplification of genomic DNA from single pollen grains of trees.*AngewandteBotanik.***72**:10-13.
29. Halldén C., Hansen M., Nilsson N.O.,Hjerdin A. andSäll T. (1996) Competition as a source of errors in RAPD analysis. *Theor Appl Genet.***93**:1185 1192.
30. Aziz A.N.,Jenkins J.N., McCarty J.C., Stelly D.M. and Saha S.(2013) Pollen Genotyping in Cotton for Genetic-Linkage Analysis.35th Annual University-Wide Research Symposium.Tennessee State University. Nashville, TN, USA. April 1-5, 2013.http://www.tnstate.edu/research/documents/res_sym_prog2013.pdf
31. Barnes W.M. (1999) FAQ sheet for Wayne Barnes' thermostable enzymes./Accessed May 5, 2014]: <http://barnes1.wustl.edu/~wayne/faq.wpa/>.