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ORIGINAL PAPER

Genetic mapping of the apospory-specific genomic region in *Pennisetum squamulatum* using retrotransposon-based molecular markers

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Abstract *Pennisetum squamulatum* reproduces by apomixis, a type of asexual reproduction through seeds. Apomixis in *P. squamulatum* is transmitted as a dominant Mendelian trait, and a genomic region, the apospory-specific genomic region (ASGR), is sufficient for inheritance of the trait. The ASGR is physically large (>50 Mb), highly heterochromatic, hemizygous, and recombinationally suppressed. These characteristics have hindered high-resolution genetic mapping and map-based cloning of apomixis genes. In this study, the long terminal repeat (LTR) regions of ASGR-abundant retrotransposons in the genome of *P. squamulatum* and ASGR-linked bacterial artificial chromosome clones were identified and sequenced for designing LTR-specific primers. Two hundred and ninety single-dose sequence specific amplified polymorphism (SSAP) markers were generated from 38 primer combinations. The SSAP markers combined with two previous ASGR-mapped markers were used for genetic linkage analysis and construction of a genetic map resulting in the formation of 27 linkage groups at LOD 10, one of which contained >60% of the SSAP markers. After removing identical markers (identical band scoring) on the largest linkage group, 46 markers were finally used for genetic mapping at LOD 10. The markers distributed across 10 different loci covering 19 cM; however, 45 markers were distributed within 9 cM. Six markers were recovered and sequenced. Five markers were successfully converted into sequence characterized amplified regions (SCARs). Segregation of SCAR markers was not always consistent with the SSAP markers of origin suggesting a greater level of error in the SSAP map resulting in an inflated map distance for the ASGR. One SCAR marker (Pst 56-1205-400) detected expression of an ASGR retrotransposon in root, anther, leaf and ovary of *P. squamulatum*, although sequencing of the RT-PCR product failed to find a functional open reading frame for the transcript.

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Introduction

Although sexual reproduction is ubiquitous in the plant kingdom, asexual reproduction through seeds (apomixis) predominates in some angiosperm species (Asker and Jerling 1992; Nogler 1984). Apomictic plants can produce progenies that are genetically identical to the maternal parent. This feature brings huge potential value to apomixis in agricultural and evolutionary research (Meeûs et al. 2007; Spillane et al. 2001, 2004; van Dijk and van Damme 2000). For example, apomixis preserves heterosis into the next generation of a hybrid, a feature that could dramatically reduce the cost of hybrid seed production. Apomixis is rarely found in crop species although it has been demonstrated to be relatively prevalent in angiosperms (Bicknell and Koltunow 2004; Carman 1997; Ozias-Akins 2006). Extensive efforts have been undertaken to introduce the apomixis trait to crop species from their wild relatives. The introgression of apomixis into related crop species is

hindered by a variety of factors such as incompatibility between pollen donor and recipient or hybrid pollen sterility (Ozias-Akins et al. 2003) and endosperm imbalance in the new hybrids (Scott 2007; Spillane et al. 2004). While no apomictic grain crops have been released, apomixis has been introgressed from *Pennisetum squamulatum* into *Pennisetum glaucum* (pearl millet) (Dujardin and Hanna 1989) and apomictic maize-*Tripsacum* hybrids have been developed (Grimanelli et al. 1995; Spillane et al. 2001). An alternative approach to producing apomictic crops might be to transfer to a sexual plant one or more well-characterized genes known to confer the trait of apomixis. The recent exploration of molecular mechanisms underlying apomixis might eventually enable the application of apomixis to crop breeding (Grimanelli et al. 2001; Grossniklaus et al. 2001; Koltunow et al. 1995; Ozias-Akins 2006).

In *P. squamulatum*, apospory has been shown to be transmitted as a single large (>50 Mb) chromosomal block named the apospory-specific genomic region (ASGR) (Akiyama et al. 2004; Goel et al. 2003; Ozias-Akins et al. 1998), unlike in some species such as *Taraxacum officinale* (van Dijk et al. 1999), *Poa pratensis* (Albertini et al. 2001), *Erigeron annuus* (Noyes and Rieseberg 2000) and *Hieracium caespitosum* (Catanach et al. 2006), where the formation of unreduced embryo sacs and parthenogenesis were confirmed to be controlled by independent loci. Further characterization of the ASGR showed that it is hemizygous, highly heterochromatic and abundant in repetitive elements such as *Opie-2*-like retrotransposons (Conner et al. 2008; Akiyama et al. 2004; Goel et al. 2003). Maternal apomeiosis and suppressed recombination in the ASGR hamper genetic mapping and map-based cloning. Although recombination-based high-resolution mapping is unlikely to be successful, physical mapping of the 50-Mb region could be accomplished if it could be saturated with molecular markers. To date, only 12 SCAR and 7 AFLP markers have been mapped to the ASGR. We sought to test the ability of the long terminal repeat (LTR) retrotransposons abundant in the ASGR to provide a unique resource for molecular marker development.

Retrotransposons, also called type I mobile genetic elements, are ubiquitous in plants and constitute a major portion of their genome (Flavell et al. 1992; Kumar and Bennetzen 1999; Suoniemi et al. 1998; Voytas et al. 1992). Depending on the presence or absence of long terminal repeats (LTR), retrotransposons are classified into two groups, LTR and non-LTR retrotransposons, respectively (Kumar and Bennetzen 1999). In plants, LTR retrotransposons appear to be the most abundant and the most transcriptionally active (Arabidopsis Genome Initiative 2000; The Rice Chromosome 10 Sequencing Consortium 2003). Their ubiquity in plants, wide dispersion on all chromosomes and activity in creating genomic diversity make them ideal for use as molecular markers (Kumar and Hirochika 2001; Schulman et al. 2004). The SSAP (sequence specific amplified polymorphism) technique was modified from AFLP (amplified fragment length polymorphism) (Vos et al. 1995; Waugh et al. 1997), in which one AFLP adapter primer for selective amplification was replaced with a LTR-specific primer. SSAP has been shown to be more efficient than other marker systems such as AFLP, selective amplification of microsatellite polymorphic loci (SAMPL) and simple sequence repeats (SSR) (Ellis et al. 1998; Lou and Chen 2007; Porceddu et al. 2002b; Tam et al. 2005; Waugh et al. 1997). To date, SSAP has been used for genetic linkage and diversity analysis in barley (Waugh et al. 1997), pea (Ellis et al. 1998; Pearce et al. 2000; Schneider et al. 1999), chickpea (Sant et al. 2000), oat (Yu and Wise 2000), alfalfa (Porceddu et al. 2002b), wheat (Queen et al. 2004), *Lotus* (Madsen et al. 2005), *Iris* (Bouck et al. 2005), lettuce (Syed et al. 2006), apple (Venturi et al. 2006), potato (Lightbourn et al. 2007), cucurbit (Lou and Chen 2007), blue agave (Bousios et al. 2007), Narbon vetch (*Vicia narbonensis*) (Sanz et al. 2007), and tomato and pepper (Tam et al. 2005). In the present study, we characterized the LTR region of a particularly abundant retrotransposon in the ASGR and used this sequence information to develop numerous apomixis-linked markers that will augment the toolbox for construction of a physical map of the ASGR in *P. squamulatum*.

Materials and methods

Plant materials and trait screening

Eighty-nine individuals from a cross between induced tetraploid pearl millet (*P. glaucum*) ($2n = 4x = 28$) and *P. squamulatum* (Ps26, PI 319196, $2n = 56$) (Goel et al. 2006) were used for molecular mapping. An apomictic individual (BC_7 line 58) from the fourth backcross between BC_3 (Dujardin and Hanna 1989) and tetraploid pearl millet also was included. Plants were characterized for mode of reproduction by clearing of ovules in methyl salicylate (Young et al. 1979) and for marker phenotype using two ASGR-linked sequence characterized amplified region (SCAR) markers (UGT197 and Q8M) (Ozias-Akins et al. 1998).

Nucleic acid extraction and purification

Genomic DNA was extracted as previously described (Ozias-Akins et al. 1993). Plasmid DNA was extracted and purified with the QIAGEN plasmid mini kit following the manufacturer's instructions (QIAGEN, Valencia, CA, USA). PCR products for TA cloning were directly purified with QIAquick PCR purification kit or gel-purified with

QIAquick gel extraction kit (QIAGEN, USA). Anther, root, leaf, and ovary RNAs were isolated with the RNeasy mini kit (QIAGEN, USA). Ovaries were collected under a microscope from heads of Ps26 at D0 (anthers beginning to exsert but prior to pollen shed) and D1 (1 day after pollination). DNA quantification was conducted with the fluorocount microplate fluorometer using Hoechst 33258 (Packard, Meriden, CT, USA) following the instruction of molecular cloning protocols (Sambrook and Russell 2001), while RNA was quantified with RiboGreen® RNA Quantitation Kit following the manufacturer's protocol (Molecular Probes, Eugene, OR, USA).

Southern blotting

Genomic DNA (8 µg) was digested overnight with 50 units of *Hin*dIII restriction enzyme (New England Biolabs, Beverly, MA) in a 30 µl reaction at 37°C. Fragments were separated on a 0.8% agarose gel (Invitrogen, USA) in 1× TBE buffer overnight at 2 V/cm and transferred to Genescreen Plus nylon membranes (NEN Life Sciences, Boston, MA) using the alkaline transfer method (Sambrook and Russell 2001). Products amplified from P800 with primers 898/901 (Table 1) were purified with QIAquick gel extraction kit (QIAGEN, Valencia, CA) and labeled with α-^{32}P-dCTP using a random priming DNA labeling kit (Roche Applied Science, Indianapolis, IN, USA). The membrane was prehybridized in 30 ml hybridization buffer (6 × SSC, 1% SDS, 100 µg/ml salmon sperm DNA) at 65°C overnight. Hybridization was conducted at 65°C overnight followed by four washes at the same temperature for 15 min each with the following buffers: (1) 2× SSC, 0.1% SDS; (2) 1× SSC, 0.1% SDS; (3) 0.5× SSC, 0.1% SDS; and (4) 0.1× SSC, 0.1% SDS. The membrane was exposed with the Cyclone Imaging System (Packard, Meriden, CT).

Generation of additional LTR sequence from the ASGR

Individual sequences, generated from random and targeted sequencing of the ASGR-BAC P800 (process detailed in Conner et al. 2008), were assembled by Phrap. A probe derived from sequence AY375366 (Akiyama et al. 2004) was used to identify clones for targeted sequencing. A BlastN analysis against the nucleotide collection database at the NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) was conducted with the sequence contigs. Sequence contigs showing similarity to LTR-retrotransposons were re-analyzed for significant similarity ($e < 10^{-4}$) to Ofovin, a LTR-retrotransposon from a pearl millet BAC clone (AF488414). Seven sequence contigs were manually assembled into an artificial P800 sequence contig and used for comparison to one complete copy of the Ofovin retrotransposon (AF488414, nucleotides 23126–34367) using Artemis Comparison Tools (ACT, http://www.sanger.ac.uk/Software/ACT).

The LTR region of one complete copy of Ofovin, Ofovin_311G2-1 was identified using Dot Matrix analysis (Vector NTI, Invitrogen, Carlsbad, CA), and a web-based program, LTR_FINDER v1.03 (http://tlife.fudan.edu.cn/ltr_finder). The LTR of Ofovin was aligned with sequence contigs of P800 using Bl2seq. ClustalX was used to align one contig from P800, CTG35-2-33, with the four copies of Ofovin LTRs from the pearl millet BAC clone. These four copies of Ofovin LTRs are from an incomplete copy of Ofovin (AF488414, nucleotides 6559–7753), a complete copy of Ofovin (AF488414, nucleotides 23126–24943, 32250–34367), and a solo Ofovin LTR (AF488414, nucleotides 100205–101167).

Primer set 882/883 (Table 1), based on the conserved regions between CTG35-2-33 and Ofovin LTRs, was used to isolate LTR sequences from the genome of Ps26 and

Table 1 LTR primers used in this study

Primer ID	Primer sequence	Source of sequence/purpose
882	GCAGGTGAAGAACAAGCG	Conserved region between CTG35-2-33 and 4 copies of Ofovin LTRs used for isolation of LTR sequences from P. *squamulatum* and ASGR-BAC clones
883	AGTGAATGGGAGCCGATAA	Conserved region between CTG35-2-33 and 4 copies of Ofovin LTRs used for isolation of LTR sequences from P. *squamulatum* and ASGR-linked BAC clones
898	GAGACAACGTGGACATGGAG	Specific region of CTG35-2-33 compared with 4 copies of Ofovin LTRs
901	AGTGCTAGCCTATGGAGAAA	Specific region of CTG35-2-33 compared with 4 copies of Ofovin LTRs
1086	GTTGTTAGCCGCCACCTTGC	IRDye 700 labeled LTR-primer from ASGR-BAC group
1087	TTCGCGCTTGTTCTTCACCTGC	IRDye 700 labeled LTR-primer from the conserved region of all sequences
1153	ACTTCATCTTGCTGTTCTTGCCACCAT	IRDye 700 labeled LTR-primer from Ofovin group
1154	GTAGCCTCCGCCAATCACCACTCAT	IRDye 700 labeled LTR-primer from Ofovin group
1155	AGCCTACTTGCCTCTCCACT	IRDye 700 labeled LTR-primer from ASGR-BAC group
1156	ATTTTTATCGGCTCCCATTCACT	IRDye 700 labeled LTR- primer from the conserved region of all sequences

Table 2 Number of single-dose markers generated from different LTR-specific/*Mse*I primer combinations in *Eco*RI/*Mse*I–LTR/*Mse*I SSAP and *Pst*I/*Mse*I–LTR/*Mse*I SSAP

Primers	Digestion	1080[c] GT[d]	1102 GC	1103 GA	1104 GG	1105 CA	1106 CT	1107 CG	1135 CC	1136 TA	1137 TC	1138 TG	1139 TT	1140 AC	1141 AG	1142 AT	1143 AA
E1086[a]	*Eco*RI/*Mse*I	12	12	4	6	6	3	7	5	9	6	5	0	7	2	2	3
E1155	*Eco*RI/*Mse*I	7	5	1	6	2	1	5	4	4	0	0	0	6	1	4	0
P1086[b]	*Pst*I/*Mse*I	11	5	11	10	10	3	7	5	12	7	6	0	6	3	2	2
P1155	*Pst*I/*Mse*I	6	5	2	4	3	2	3	4	4	2	3	0	4	na[e]	na	na

[a] *Eco*RI/*Mse*I enzymes were used for digestion, and LTR-specific primers and *Mse*I adapter primers plus two selective nucleotides were used for selective amplification

[b] *Pst*I/*Mse*I enzymes were used for digestion, and LTR-specific primers and *Mse*I adapter primers plus two selective nucleotides were used for selective amplification

[c] ID of *Mse*I adapter primer plus two selective nucleotides

[d] Selective nucleotides added to the *Mse*I adapter primer

[e] Not assayed

previously characterized (Akiyama et al. 2004; Goel et al. 2006; Roche et al. 2002) ASGR-linked BACs (P602, P702, P703, P800, P900). Amplified Ps26 products were ligated into pGEM-Easy TA cloning vector (Promega, Madison, WI, USA) and transformed into *E. coli* JM109 competent cells (Promega, USA) via the heat-shock method. Based on insert sizes and *Alu*I digestion patterns, 47 colonies were initially chosen for sequencing. Seven fragments from 5 BACs were gel purified and directly sequenced. Sequencing of inserts and PCR products was carried out with a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA). Sequences were deposited in GenBank (FJ267592-FJ267606).

Alignments were conducted with all generated LTR sequences, CTG35-2-33 and one Ofovin LTR from the complete copy of Ofovin (AF488414, nucleotides 23126-24943) using ClustalX. The dendrogram and alignment results were viewed with TREEVIEW 2.0. Six LTR-specific primers were generated from the alignments (Table 1). LTR-specific primers were labeled with fluorescent IRDye 700 (MWG-BIOTECH**,** High Point, NC, USA) for SSAP.

Development of sequence specific amplified polymorphism

EcoRI/MseI-LTR/MseI SSAP

The procedure used for this type of SSAP has been described by Porceddu et al. (2002b). Briefly, digestion and ligation of genomic DNA (500 ng) was conducted for 4 h at 37°C in a reaction volume of 50 µl, including 5 U of each restriction enzyme (*Eco*RI and *Mse*I; New England Biolabs, Beverly, MA, USA), 1 U of T4 DNA ligase (Promega, USA), 10 mM ATP, 50 pmol *Mse*I adaptor, and 5 pmol *Eco*RI adaptor in RL buffer (20 mM Tris-acetate, 20 mM magnesium acetate, 100 mM potassium acetate, 5 mM DTT, 0.05 µg/µl BSA). Pre-amplification was performed in a 50 µl PCR reaction containing 5 µl of tenfold diluted (digested and ligated) DNA, 30 ng of the *Eco*RI and *Mse*I adaptor primers, 10 mM dNTPs, 0.5 U *Taq* DNA polymerase (New England Biolab, USA) in 1× PCR buffer (10 mM Tris-HCL, 50 mM KCl, 1.5 mM $MgCl_2$). The pre-amplified PCR product was diluted tenfold and 5 µl of the diluted template was used for a 20 µl selective amplification. The reaction mixture was the same as the pre-amplification reaction except that an IRDye-labeled LTR-specific primer and *Mse*I + 2 primers (30 ng each) were used (Tables 1, 2). The cycling conditions for pre-amplifications and selective amplifications were initiated with one cycle of 45 s at 94°C, 30 s at 65°C, 1 min at 72°C, followed by 13 cycles of a touch-down profile with the annealing temperature decreased at a rate of 0.7°C/cycle, and 20 cycles at a constant annealing temperature of 55.9°C. A final extension step at 72°C for 7 min was carried out. After selective amplifications, 10 µl of blue stop solution (*LI-COR* Bioscience, Lincoln, Nebraska, USA) was added to each sample. Samples were denatured at 94°C for 5 min and then immediately placed on ice. A 6.5% polyacrylamide gel was pre-run at 1,500 V for 20 min on a *LI-COR* 4300 DNA Analyzer (*LI-COR* Bioscience, USA). Samples (0.5 µl each) were loaded onto the gel and then run for 3.5 h under the same parameters.

Initially, 10 F_1 plants (5 apomicts and 5 sexual plants) and the two parents were used to test 96 primer pairs (16 *Mse*I + 2 × 6 LTR-primers). Thirty-two primer pairs producing the largest number of informative fragments were used for screening 89 F_1 individuals plus two parents.

Table 3 Number of single-dose markers generated from different LTR-specific/*Pst*I primer combinations in *Pst*I/*Mse*I–LTR/*Pst*I SSAP

Primers	Digestion	1086	1087	1153	1155	1156
Pst1205[a,b]	*Pst*I/*Mse*I	–[c]	–	–	3	3
Pst1206	*Pst*I/*Mse*I	na[d]	2	na	5	2

[a] *Pst*I/*Mse*I enzymes were used for digestion, and LTR-specific primers and *Pst*I adapter primers plus one selective nucleotide were used for selective amplification

[b] Pst1205, GACTGCGTACATGCAG + A; Pst1206, GACTGCGTACATGCAG + C

[c] Not an informative primer combination

[d] Not assayed

PstI/MseI-LTR/MseI SSAP

*Pst*I/*Mse*I was also tested for digestion with the same procedure described above except that *Pst*I/*Mse*I instead of *Eco*RI/*Mse*I adapter primers were used for pre-amplification (Tables 1, 2). Primer pairs used in *Eco*RI/*Mse*I-LTR/*Mse*I SSAP were applied to this type of SSAP except the noted ones in Table 2.

PstI/MseI-LTR/PstI SSAP

LTR/*Pst*I primer combinations were applied to selective amplifications instead of LTR/*Mse*I primer pairs as described above (Tables 1, 3). Ten F_1 plants (6 apomicts and 4 sexual plants) and the two parents were used to test 24 primer pairs. Six out of eight informative primer combinations were finally used to screen the F_1 population plus two parents.

Linkage analysis and map integration

Only single-dose markers were used for linkage analysis. Bands on the gel image were manually scored as 1 (band present) or 0 (band absent). Goodness-of-fit was assessed using χ^2 at a significance level of 5% to identify single-dose markers by their 1:1 segregation ratio that were present in *P. squamulatum* and absent in pearl millet and segregating among the progeny. To analyze the scored markers, segregation distortion tests and linkage analyses were performed by using JoinMap 3.0 (Van Ooijen and Voorrips 2001) with the parameters set for BC_1-derived progeny as the mapping of single-dose markers in polyploids is equivalent to backcross (BC) mapping in diploids. SSAP markers combined with one previous AFLP marker (PQ355) and one SCAR marker (UGT197) were used for linkage analysis (Goel et al. 2006). Map distances expressed in centiMorgans (cM) were estimated based on the recombination fraction using the Kosambi function. Marker grouping was initiated with a logarithm of odds (LOD) score of 10, and the parameter for showing weak linkage was changed from a default value of 0.45 to a more stringent value of 0.35. Genetic mapping was limited to only the largest linkage group containing the ASGR-linked AFLP and SCAR markers. Parameters used for marker ordering within a linkage group were Rec = 0.30, LOD = 10.0 and Jump = 5.0.

Marker recovery and SCAR development

Fluorescence-labeled amplified PCR products (5–7 µl) were separated on a 6.5% polyacrylamide gel and specific fragments were excised using Odyssey infrared imaging system (*LI-COR* Bioscience, USA). Gel slices were immersed in 25 µl TE buffer (pH 8.0), subjected to three cycles of freezing and thawing and pelleted by centrifuging at 15,000*g* for 25 min. An aliquot of 2 µl was used for a 20-µl selective amplification reaction. The PCR conditions were 3 min at 94°C; 40 cycles of 30 s at 94°C, 30 s at 56°C, 1 min at 72°C; and 7 min at 72°C for the final extension. Integrity of the recovered fragments was confirmed first by running a *LI-COR* gel. After confirmation, another round of PCR with corresponding non-labeled LTR-specific primers was performed with the same PCR profile described above, separated on a 1.5% agarose gel, and purified with the QIAquick gel extraction kit (QIAGEN, USA). Gel-purified fragments were ligated with the PCR®4-TOPO® vector (Invitrogen, USA) and transformed into one shot TOP10 *E. coli* DH5α following the manufacturer's instructions (Invitrogen, USA). Sequencing was performed by the Riverbend Research Lab Sequencing and Synthesis Facility, University of Georgia, Athens, GA. Sequences were deposited in GenBank (FJ267607-FJ267612). Sequence analysis and primer design were conducted with Vector NTI/Contig Express 7.0 (Invitrogen, USA).

Expression analysis of the ASGR retrotransposon by RT-PCR

SCAR primers derived from SSAP marker Pst56-1205-400, which completely co-segregated with apomixis, were used for RT-PCR because this marker showed similarity to the encoding region of one rice retrotransposon. Approximately 1.2 µg of root, anther, leaf and ovary (DAP 0 and DAP 1) total RNA were used for cDNA synthesis with the SuperScript II first-strand synthesis system (Invitrogen, USA). Controls without reverse transcriptase were included for all samples. A 2-µl aliquot of cDNA was used in a 20-µl RT-PCR reaction with the following PCR conditions: initial denaturation step at 94°C for 3 min, followed by 38 cycles of 94°C for 30 s, 55°C 30 s and 72°C for 1 min. PCR products were separated on a 2% agarose gel.

Results

Identification and sequencing of LTRs

Previous analysis of sequences from ASGR-linked BAC P800 had identified some with high protein similarity to an *Opie-2*-like retrotransposon from rice (Akiyama et al. 2004). Sequencing of additional P800 subclones containing similarity to this *Opie-2*-like retrotransposon was conducted for this study. Seven sequence contigs were generated which had similarity to the Ofovin retrotransposons in a pearl millet (*P. glaucum*) BAC clone (AF488414) (Fig. 1a). Pearl millet is a sexually reproductive crop species related to *P. squamulatum*. Ofovin is a *Ty-1/copia* type mobile element, which has high protein similarity with another *Opie-2*-like retrotransposon from rice (AAN60494, $E = 0$). Two P800 sequence contigs showed similarity to both ends of Ofovin_311G2-1, a complete Ofovin retrotransposon in the pearl millet BAC (Fig. 1a). Dot matrix analysis conducted with Ofovin_311G2-1 identified the direct repeats characteristic of LTR regions (Fig. 1b). The first and last 1,818 bp shared 99.78% identity, more clearly delineating the 5′ and 3′ LTRs. Subsequent characterization of 311G2-1 using LTR_FINDER further confirmed the Ofovin LTR regions which terminated with TG at the 5′ end and CA at the 3′ end (data not shown).

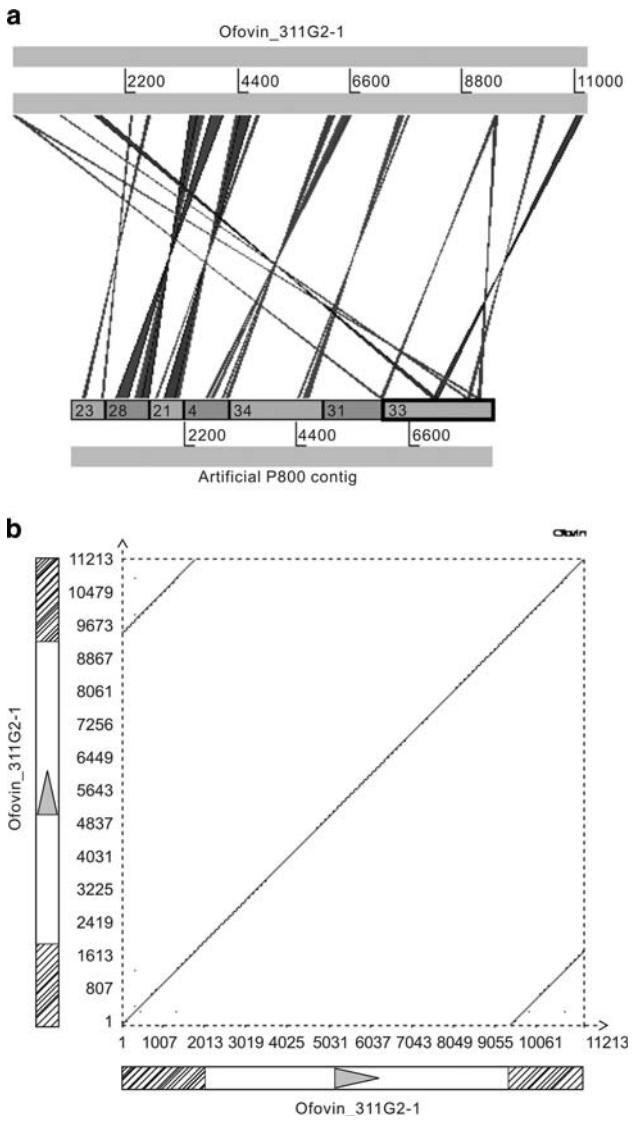

Fig. 1 **a** Nucleotide comparison of Ofovin_311G2-1 with an artificial ASGR-BAC P800 sequence contig. *Numbers within the bars* represent the last two digits of different sequence contigs, for example, 33 stands for 35-2-33; **b** Dot matrix analysis of Ofovin_311G2-1. LTRs are indicated with *hatched boxes*. *Arrows* show the orientation of full-length Ofovin_311G2-1

The longest P800 contig, CTG35-2-33, showing highest similarity to Ofovin LTRs was chosen for further study. One set of primers (882/883) was subsequently designed based on the conserved regions between this contig and four copies of Ofovin LTRs (Table 1). This amplicon spans ~88% of the Ofovin LTR and was used to obtain additional LTR sequences from multiple members of the *Opie-2*-like retrotransposon family from Ps26. Thirty-three out of 36 end sequences of LTR clones generated from the Ps26 genome were clustered into two distinct groups. Sixteen Ps26-LTR sequences clustered with CTG35-2-33 while 17 other Ps26-LTR sequences clustered with the Ofovin LTR (data not shown). Eight Ps26 LTR clones (five from the CTG35-2-33 cluster and three from the Ofovin cluster) were selected for complete sequencing. Seven products amplified with primers 882/883 from five ASGR-mapped BACs also were completely sequenced. The three LTR clones from genomic DNA that had clustered with Ofovin remained tightly clustered when the complete sequence was analyzed (Fig. 2). Southern blot analysis of a 478-bp fragment, derived from the p800 BAC clone using primers 898/901 located within the 882/883 derived sequence, was done to determine ASGR specificity of this LTR region (Fig. 3). All genotypes compared, Ps26, IA4X and BC_7 line 58, showed both strong and weak hybridization signals throughout their respective lanes. No ASGR-specific band could be identified when comparing hybridization patterns of the two apomicts to the single sexual genotype. Therefore, clustering was used to inform primer design where six LTR primers for SSAP were finally generated: two from the alignment of all sequences, two from the Ofovin LTR group, and two from the ASGR-linked BAC group (Table 1; Fig. 2). LTR-primers were designed from the sequence region close to either the 5′ end or 3′ end to maximize the proportion of genomic sequence flanking the LTR. Restriction sites of enzymes *Eco*RI, *Pst*I and *Mse*I were avoided during primer design.

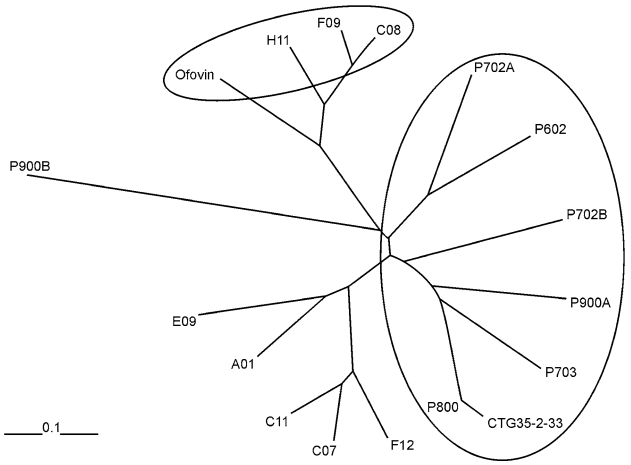

Fig. 2 Analysis of LTR sequences generated from the *P. squamulatum* genome and ASGR-linked BACs. LTR primers were designed based on the alignment of all sequences within the two *circled* groups. Ofovin: a LTR of Ofovin_311G2-1 (AF488414); CTG35-2-33: a sequence contig of ASGR-BAC P800. P602-P900: sequences from ASGR-BACs generated with primers 882/883. All other sequences were from randomly picked clones generated from Ps26 genomic DNA amplified with primers 882/883

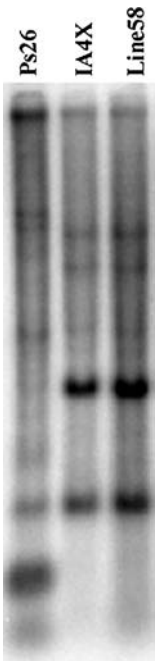

Fig. 3 Southern blot to test for specificity of a portion of the LTR. Ps26 and Line58 are apomictic, both having the ASGR-carrier chromosome; IA4X is sexual

Generation of sequence specific amplified polymorphism

EcoRI/MseI–LTR/MseI SSAP

In this type of SSAP, *Eco*RI/*Mse*I enzymes were used for digestion and LTR-specific primers paired with *Mse*I adapter primers plus two selective nucleotides were used for selective amplification. Initially, the *Mse*I/*Eco*RI adapter primer, without any selective nucleotide and with one selective nucleotide, was tested for pre-amplification. For selective amplification, *Mse*I +1, +2, or +3 selective nucleotides were tested. Based on the scorable polymorphisms and reproducibility of reactions, the *Mse*I/*Eco*RI + 0 selective nucleotide for pre-amplification and *LTR*/*Mse*I + 2 selective nucleotides for selective amplification were chosen. Ninety-six primers (16 *Mse*I + 2 selective nucleotide primers by 6 LTR primers) were screened with five sexual and five apomictic F_1 plants plus the two parental plants, PS26 and IA4X, to determine the most informative combinations to identify ASGR-linked SSAP markers. Thirty-two primer combinations were chosen to screen the 89-individual F_1 population (52 apomicts, 37 sexuals) along with two parents. One hundred and thirty-five single-dose markers were generated (Table 2). Primer combinations producing the most single-dose markers were formed by all 16 *Mse*I + 2 primers paired with only two LTR-specific primers (1086 and 1155), both of which were from the ASGR-linked BAC group. An average of 4.2 single-dose markers was produced per primer combination (for 32 primer combinations) with a range from 0 to 12. Ninety-three of these markers (~68.9%) were shown to be closely linked with apomixis, and when the missing data were eliminated, 78 markers (~57.8%) were shown to fully co-segregate with apomixis.

PstI/MseI−LTR/MseI SSAP

*Pst*I/*Mse*I enzymes were used for digestion in *Pst*I/*Mse*I–LTR/*Mse*I SSAP. Pre-amplifications were obtained with *Pst*I/*Mse*I adapter primers without any selective nucleotides, while *Mse*I adapter primers plus two selective nucleotides with LTR-specific primers were used for selective amplifications. One hundred and forty-two single-dose markers were generated from 29 primer combinations (Table 2). Eighty-five markers (~59%) were linked with apomixis, and 73 (~51%) were completely linked with the trait when the missing data of F_1 individuals for some markers was not considered. Approximately 4.9 single-dose markers per primer combination were generated with a range from 0 to 11.

PstI/MseI–LTR/PstI SSAP

This type of SSAP is the same as *Pst*I/*Mse*I–LTR/*Mse*I SSAP except that *Pst*I adapter primers plus one selective nucleotide were used with LTR-specific primers for selective amplifications. Ten F_1 plants (4 sexual, 6 apomictic) and two parental plants were used for screening 24 primer combinations (Table 3 and data not shown). Eight informative primer combinations were obtained using five LTR-specific primers, although the average number of

polymorphic bands per primer combination was less. Thirteen single-dose markers were generated from six primer combinations. Six markers were shown to be closely linked with apomixis, two of which completely co-segregated with the trait (Table 3).

Genetic mapping of the ASGR in *P. squamulatum* using retrotransposon-based markers

A total of 290 SSAP markers combined with a previous SCAR marker UGT197 and an AFLP marker PQ355 was used for genetic linkage analysis. Four individuals which showed missing genotype data for over 20 (5%) markers and two individuals showing odd banding patterns in all images were excluded from the dataset, leaving data from 83 F_1 individuals to construct the map. Twenty-seven linkage groups were formed at a LOD score of 10, and 38 markers (13%) were unlinked. The largest group contained 186 (63.7%) of the markers. The second largest group contained six. No group of markers was found which segregated exclusively with the sexual F_1 genotypes. Joinmap automatically excluded 141 identical markers (identical band scoring) from the dataset leaving 46 markers to form the largest group at LOD10 (Table S1). Ninety-five of the 141 excluded markers had a segregation pattern identical to SCAR UGT197. Using the dataset with excluded markers, the LOD threshold was dropped from 10 to 5 in a stepwise manner, resulting in one additional marker (P86-1105-334 at a LOD value <9.0) in the largest linkage group.

The group containing 46 markers at LOD 10 was used for construction of a genetic linkage map using a stringent set of parameters (Rec = 0.30, LOD = 10.0 and Jump = 5.0) (Fig. 4). The 46 markers were distributed onto ten different loci and covered 19 cM. Forty-five markers spanned 9 cM. The ASGR-linked SCAR UGT197 mapped with 21 other co-segregating SSAP markers. The remaining 23 SSAP markers including PQ355 were distributed along eight different loci flanking the UGT197 locus (four loci each side) (Fig. 4). PQ355 is an AFLP marker shown to recombine in two out of 193 F_1 individuals and has been cytogenetically mapped to a position more proximal to the centromere of the ASGR-carrier chromosome than UGT197 (Goel et al. 2006). As a low frequency of genotyping error is unavoidable in AFLP-based marker data (Remington et al. 1999; Bonin et al. 2004; Pompanon et al. 2005) and because recombination around the ASGR previously has been observed only on the centromeric side of SCAR UGT197, we suspected that some of the putative recombination events could be erroneous. In order to confirm recombination, SCAR development was pursued for several markers.

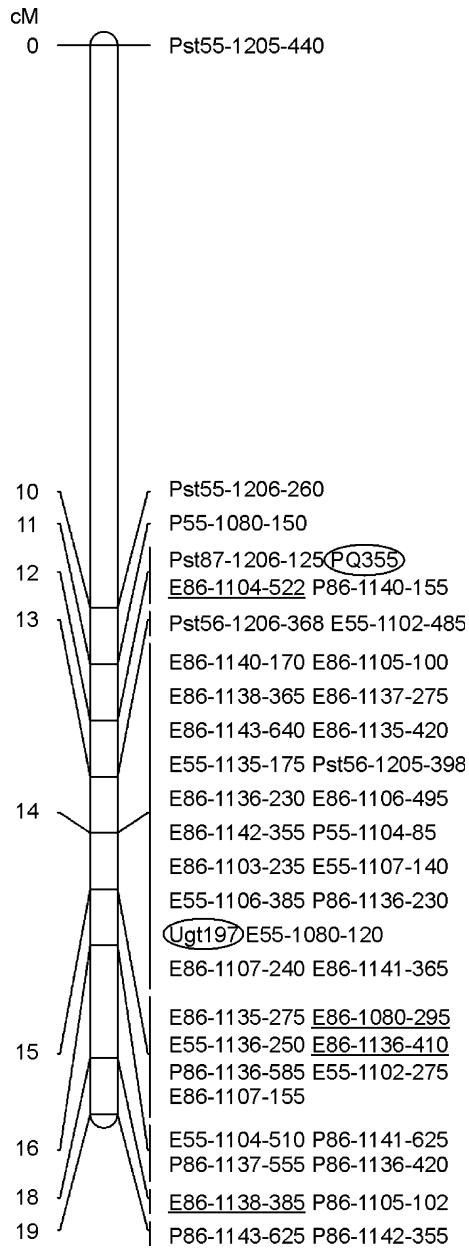

Fig. 4 Genetic linkage map of ASGR SSAP markers. The *letters in marker* IDs (E, P and Pst) indicate that the marker was generated from *Eco*RI/*Mse*I-LTR/*Mse*I, *Pst*I/*Mse*I-LTR/*Mse*I or *Pst*I/*Mse*I–LTR/*Pst*I SSAP, respectively. The first two digits correspond to the last two digits of LTR-specific primers; the four digits in the middle indicate the ID of *Mse*I + 2 primers or *Pst*I + 1 primers; the last two or three digits indicate the estimated band size. *Circled markers* are previously published and cytogenetically mapped to the ASGR. *Markers underlined* were chosen for SCAR development

Marker recovery and SCAR development

Combining information from the genetic map constructed in this study and the physical map described by Goel et al. (2006), six SSAP markers were selected for potential SCAR development. Five showed recombination in F_1

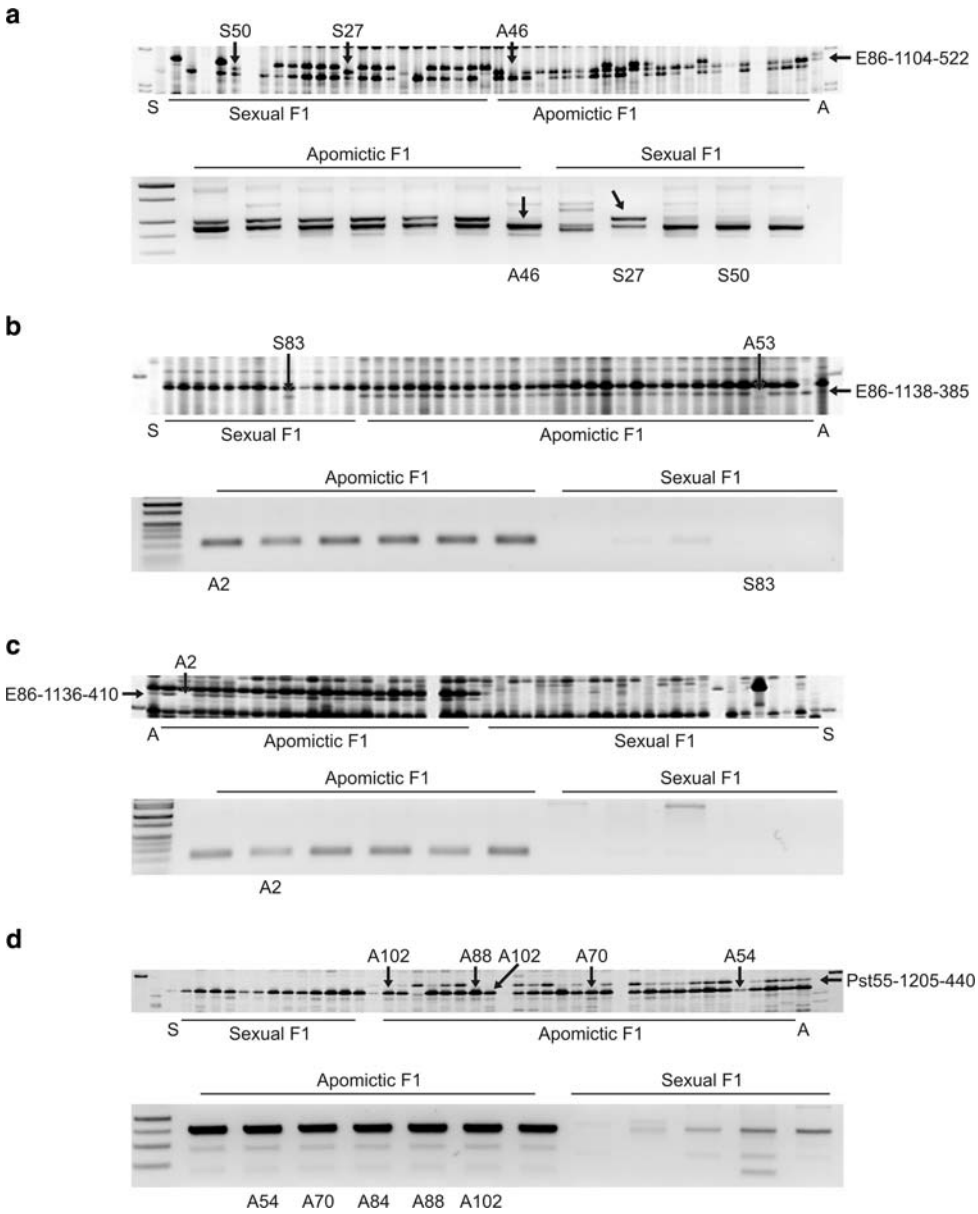

Fig. 5 **a** *Upper panel* partial profile of a SSAP marker (E86-1104-522) showing recombination in three F_1 plants (S50, S27 and A46); *lower panel* SCAR marker (E86-1104-522) was used for screening 11 F_1 plants including the three recombinants. Recombination was confirmed in 2 of 3 F_1 plants with this SCAR (indicated by *arrows*). S, IA4X; A, Ps26; **b** *upper panel* partial profile of a SSAP marker E86-1138-385 which showed recombination in three F_1 plants [A53, S83 and A2 (not shown)]; *lower panel* SCAR marker E86-1138-385 was used for screening 11 F_1 plants including the three putative recombinant F_1 plants (A2, A53 and S83). No recombination was identified in the three F_1 plants with this SCAR; **c** *upper panel* partial profile of a SSAP marker (E86-1136-410) showing recombination in one F_1 plant (A2); *lower panel* SCAR marker E86-1136-410 was used for screening 11 F_1 plants including the recombinant F_1 plant (A2). No recombination was identified in the F_1 plant with this SCAR; **d** *upper panel* partial profile of a SSAP marker (Pst55-1205-440) showing recombination in 11 F_1 plants (only 5 plants shown here); *lower panel* SCAR marker Pst55-1205-440 was used for screening 12 F_1 plants including 5 recombinants. No recombination was identified in the five F_1 plants with this SCAR

plants (Figs. 4, 5a–d), while a sixth (Pst56-1205-400) generated from *Pst*I/*Mse*I–LTR/*Pst*I SSAP co-segregated with apomixis (Fig. 6a). Sequence analysis showed that five of the markers displayed similarity by BlastN to the Ofovin LTR from pearl millet BAC clone (AF488414). The marker, Pst56-1205-400, which co-segregated with apomixis, had the most significant hit to a transposable element protein in rice (*Oryza sativa*) (AAQ56333, $E = 8e\text{-}11$) when BlastX was performed. Only one of the six markers, E86-1080-295, could not be converted to a SCAR with the primer pairs chosen. SCAR markers were either dominant (present in apomictic F_1, absent in sexual F_1) or polymorphic in amplification pattern within the F_1 population (Figs. 5a–d, 6b). One SSAP marker (E86-1104-522) initially was scored as recombining in three different F_1 plants (A46, S27 and S50), while recombination of its corresponding SCAR was detected in only two plants (A46 and S27; Fig. 5a). The other three SCAR markers did not show any recombination in F_1 plants contrary to what was observed for their corresponding SSAP markers (Fig. 5b–d). Therefore, recombination was confirmed in individuals A46 and S27 with a second marker in addition to PQ355, but not for markers distal to UGT197. The potential for recombination distal to UGT197 would need to be further tested by converting other SSAP markers to SCARs and by testing on a larger population. The sequence generated from Pst56-1205-400 showed similarity by BlastX analysis to a transposable element protein (data not shown). Even though the genomic fragment did not contain a functional ORF based on the BlastX analysis, potential expression was tested by

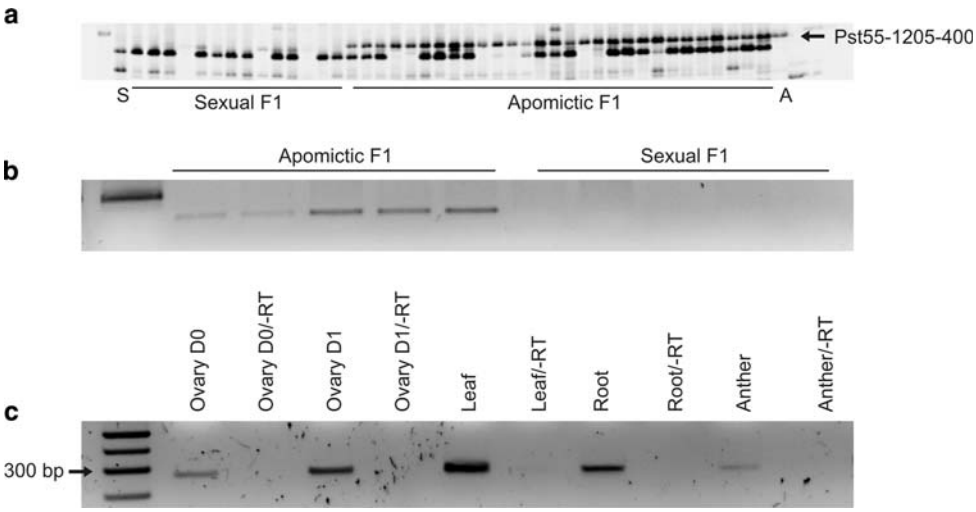

Fig. 6 **a** Partial profile of a SSAP marker, Pst56-1205-400 showing co-segregation with the ASGR; **b** SCAR marker Pst56-1205-400 was used for screening 10 F_1 individuals. **c** Detection of expression of an ASGR-specific LTR-retrotransposon using SCAR marker Pst56-1205-400. All tissues were from Ps26. *Ovary D0* ovaries at the stage of D0, i.e. anthers beginning to exsert but prior to pollen shed. *Ovary D0/-RT*, no RT control to check for DNA contamination. *Ovary D1* ovary at 1 day after pollination

RT-PCR using SCAR primers. Expression was observed in root, anther, leaf, and non-fertilized and fertilized ovaries (Fig. 6c). However, cloned and sequenced RT-PCR products were identical to the genomic SCAR fragment and did not reveal any functional open reading frame.

Discussion

Identification of LTR sequences from the ASGR

Most retrotransposon-based markers require sequence information from long terminal repeats (Kumar and Hirochika 2001; Pearce et al. 1999; Schulman et al. 2004). The method developed by Pearce et al. (1999) has been extensively used for isolating LTR sequences from *Ty1/copia* type retrotransposons in a variety of plant species (Acquadro et al. 2006; Berenyi et al. 2002; Bousios et al. 2007; Lou and Chen 2007; Pearce et al. 2000; Syed et al. 2005). The principle of this method takes advantage of the conserved RNase H motif upstream of the 3′ LTR. After digestion with a frequent-cutter enzyme, adapters are ligated to the digested genomic fragments and amplifications are conducted with a biotinylated RNaseH motif primer and adapter primers. This method, however, is limited to retrotransposons whose LTRs are within 500 bp of the RNase H motif (Pearce et al. 1999), and therefore is likely to exclude *Opie*-like elements, in which the intervening sequence between the RNase H motif to the 3′ LTR is over 1 kb. Another pitfall of this method is that the sequences recovered are not specific to a retrotransposon family, complicating the development of SSAP using a particular LTR, for example, the ASGR-abundant retrotransposon (Pearce et al. 1999). We did not attempt to sequence a full-length LTR-retrotransposon since we have not yet identified an active ASGR-LTR-retrotransposon and preliminary analysis of BAC clones based on hybridization using the LTR region suggested that the elements could be greater than 12 kb in length.

We took advantage of one complete copy of a related retrotransposon, Ofovin, from a pearl millet BAC clone (AF488414) for identification of ASGR-abundant retrotransposon LTR sequences. Ofovin is a *Ty-1/copia* type mobile element, which has high similarity with an *Opie-2* like retrotransposon from rice (AAN60494, $E = 0$). *Opie*-2-like retrotransposons were found to be abundant in the ASGR-linked BAC clones (Akiyama et al. 2004). Sequence contigs derived from ASGR-linked BAC P800 showed high similarity to another *Opie-2*-like retrotransposon from rice (NP912535, E-value $<e^{-08}$) and also shared similarity to Ofovin. Because distance between the RNase H motif and 3′LTR of Ofovin is over 1.5 kb, the method described by Pearce et al. (1999) was not deemed suitable for this study. A modified version of the method could be used if conserved regions within the LTR could be found. Although Ty-1/*copia* retrotransposons are highly heterogeneous in the plant kingdom, it was found that variations within a subgroup of a retrotransposon family were not as significant as inter-subgroups in the same family (Flavell et al. 1992, 1997; Kumar and Bennetzen 1999), suggesting that the relatively conserved LTR sequences are more likely to belong to the same subgroup of a LTR-retrotransposon. LTR sequences were successfully generated from the *P. squamulatum* genome and ASGR-linked BAC clones using primers derived from the conserved region between CTG35-2-33 and four copies of the Ofovin LTR, indicating that within the retrotransposon subgroup, conserved regions of the LTR could also be used for isolation of LTRs from different species.

Based on the nucleotide alignment, sequences isolated by PCR from genomic DNA and ASGR-linked BAC clones clustered into distinct groups. The separate clustering of

PCR-generated genomic DNA clones and the corresponding absence of a genomic PCR clone from the BAC cluster was probably due to the small number of genomic DNA clones sequenced relative to the large genome size of *P. squamulatum*. While the LTR sequences clustered into distinct groups, nucleotide variation among sequences was insufficient to identify an ASGR-LTR-specific probe based on Southern blot hybridization results. LTR sequences isolated from *P. squamulatum* and ASGR-linked BACs showed moderate similarity to pearl millet Ofovin. Whether the sequences within the Ofovin group are part of the same retrotransposon subfamily as Ofovin and whether they are related in origin are still undetermined.

Generation of sequence specific amplified polymorphisms

In this study, three types of SSAP were generated from the digestion of two different enzyme combinations, *Eco*RI/*Mse*I and *Pst*I/*Mse*I. As the ASGR is highly heterochromatic and abundant in repetitive DNA (Akiyama et al. 2004), and therefore likely to be highly methylated (Avramova 2002), we expected the DNA methylation sensitive enzyme *Pst*I to cut more frequently within euchromatic regions than heterochromatic regions which could help target the genic regions within the ASGR.

The composition of the selective bases on the *Mse*I primer did not have as strong an impact on the number of bands detected and the level of polymorphism as did the sequence of LTR primers. Markers generated in these two types of SSAP were confined to two LTR-specific primers (1155 and 1086) which originated from the ASGR-BAC sequence alignment. Given that >60% of the SSAP markers generated by these primers were linked with apomixis, this approach had unprecedented efficiency for targeting a genomic region, the ASGR. In the third type of SSAP, *Pst*I/*Mse*I–LTR/*Pst*I SSAP, LTR primers from groups other than the ASGR-BAC group also were informative, although the average number of polymorphic bands was lower than in the other two types of SSAP.

Genetic mapping of the ASGR in *P. squamulatum* using retrotransposon-based markers

Because *P. squamulatum* is a heterozygous polyploid with segregating alleles in progeny of the cross with pearl millet, the pairing behavior of chromosomes in this species is still unclear, which makes genetic mapping of the ASGR very complicated. A practical way for direct mapping of polyploids is to use single-dose markers. A single-dose marker should segregate in a 1:1 ratio in the progeny of a simplex by nulliplex cross, greatly simplifying the genetic linkage analysis for polyploids (Porceddu et al. 2002a; Wu et al. 1992). In our case, the ASGR of *P. squamulatum* has been confirmed to be hemizygous (heterozygous); therefore, a band present only in the ASGR will be inherited by approximately half of the gametes and is considered single dose. When apomictic *P. squamulatum* is crossed with sexual tetraploid pearl millet containing no ASGR, the segregation ratio for apomixis versus sexuality expected in the progeny is also approximately 1:1, although segregation distortion resulting in a deficiency of apomicts has been reported (Ozias-Akins et al. 1998).

Using the three types of SSAP, 290 single-dose markers were generated from a total of 801 polymorphic markers. Genetically, the SSAP markers were strongly clustered at the ASGR. Given that the physical size of the ASGR is known to be >50 Mb, it is likely that the large number of SSAP markers generated will not all be physically clustered in a small area but distributed around the ASGR. However, distribution of the SSAP markers across the region can only be determined after BACs containing these markers have been isolated. Clustering and nesting of retrotransposons have been reported in other genetic and physical mapping studies (Bouck et al. 2005; Boyko et al. 2002; Manninen et al. 2000; Yu and Wise 2000). Retrotransposon clusters, particularly in intergenic regions, often coincide with suppression of recombination and reflect a notable feature of the grass genome in which gene islands are embedded among repetitive elements (Dooner and He 2008; Panstruga et al. 1998).

Suppression of recombination has been observed at the apomixis "locus" of many species, reported for *P. squamulatum* (Ozias-Akins et al. 1998) and *Tripsacum dactyloides* (Grimanelli et al. 1998), and subsequently identified in diplosporous *E. annuus* (Noyes and Rieseberg 2000), aposporous *Paspalum simplex* (Labombarda et al. 2002), and *Cenchrus ciliaris* (Akiyama et al. 2005; Goel et al. 2006; Jessup et al. 2002). Suppressed recombination at the ASGR, whose position is proximal to the short arm telomere of one chromosome in *P. squamulatum,* has been described and extensively discussed (Akiyama et al. 2004; Goel et al. 2003, 2006; Ozias-Akins et al. 1998), 2003. To date, only two markers, UGT204 and PQ355, have been shown to recombine with the ASGR in *P. squamulatum*. UGT204 maps at a considerable genetic distance (~24 cM) from the ASGR (Ozias-Akins et al. 1998). PQ355 is positioned 2 cM away from the ASGR and physically mapped to a position proximal to the centromere (Goel et al. 2006). The BAC P1300 contains the recombinant marker PQ355 and hybridized not only to the ASGR-carrier chromosome but also with its apparent homolog, indicating that its hybridization pattern is not hemizygous as observed for the majority of non-recombinant BACs (Akiyama et al. 2004; Goel et al. 2003, 2006). One of the new SSAP markers (E86-1104-522), when converted to a SCAR, was confirmed to recombine and co-segregate with PQ355. Other

SSAP markers, however, failed to show recombination when converted to SCARs. SSAP marker Pst55-1205-440 was initially scored as recombining in 11 apomictic F_1s but not any sexual F_1. The SCAR developed from Pst55-1205-440 did not show any recombination in all tested F_1 plants. This result is most likely due to DNA methylation differences among the F_1s since Pst55-1205-440 originated from the *Pst*I/*Mse*I–LTR/*Pst*I SSAP in which the DNA methylation sensitive enzyme, *Pst*I, was used for digestion. It is known that wide hybridization can cause DNA methylation changes in the progenies of some crosses (Ainouche et al. 2003; Liu et al. 2004). We have observed expression differences for one apomixis candidate gene from the ASGR in F_1 hybrids compared with parental *P. squamulatum* where expression was detected in the leaves of some F_1s, but was not detectable in the leaves of the apomictic parent (unpublished data). It is possible that these expression differences are related to changes in methylation.

One SSAP marker (Pst56-1205-400), co-segregating with apomixis and successfully converted to a SCAR marker, showed sequence similarity to a *Ty-3*/*gypsy* type retrotransposon protein from rice (AAQ56333). These ASGR-specific primers identified a transcriptionally active retrotransposon. Unfortunately, analysis of the RT-PCR results revealed that a non-functional protein is being produced, and therefore it is unlikely that this SSAP fragment will allow us to isolate an actively transposing ASGR-linked element. While no evidence for transposition has been provided in this study, the transposition power of this retrotransposon could be detected by checking its presence in progeny of BC_8, an eighth backcross line between Ps26 and a tetraploid pearl millet that has been confirmed to have only a single chromosome of *P. squamulatum* (unpublished data). If transposition has occurred, the marker may no longer be ASGR specific.

In conclusion, a retrotransposon that had previously been shown to be abundant in the ASGR was demonstrated to contain sufficiently unique LTR sequence to allow SSAP marker development that efficiently targeted the ASGR. Random versus clustered distribution of these markers within the ASGR and transposition activity of transcribed members of the retrotransposon family remain to be tested.

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