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SSR markers developed for genetic mapping in flax (*Linum usitatissimum* L.)

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Abstract: Simple sequence repeats (SSRs) are useful molecular markers for genetic mapping and variety identification. A set of SSR markers was developed for use in the construction of a genetic map to identify genes involved in controlling genomic restructuring in flax (*Linum usitatissimum* L.). SSR sequences were isolated from the flax genome using a modified fast isolation by amplified fragment length polymorphism of sequences containing repeats (FIASCO) procedure. Primers were made around the SSRs, and these regions were amplified using polymerase chain reaction. Forty-two of 92 (46%) SSRs showed polymorphisms. The number of alleles for each SSR ranged from two to eight, with an average of 3.32. Polymorphism information content (PIC) was calculated for the polymorphic SSRs, with a range of 0.1049–0.8642, the average being 0.47. Several SSRs were also found informatically using genome sequence data from the flax variety Bethune. Seven of 15 (47%) were polymorphic, with an average number of alleles of 4.14, and an average PIC of 0.59. These markers add to the resources available for genetic mapping and variety identification in flax.

Keywords: simple sequence repeats, microsatellites, *Linum usitatissimum*, flax, genetic map, FIASCO

Introduction

Flax (*Linum usitatissimum* L.) is a self-pollinating annual crop grown for its fibers and oil. In addition to being important agriculturally, flax undergoes a novel and, so far, unique set of genomic changes in response to nutrient stress that can become stable and heritable.[1–8] These changes include differences in total nuclear DNA content, copies of large ribosomal ribonucleic acid (rRNA) and 5S rRNA genes, and copies of highly repetitive DNA fractions.[6–11] Another change is the insertion of a sequence termed Linum Insertion Sequence I (LIS-1), a 5.7 kilobase stretch of DNA, which appears to be assembled from short sequences scattered throughout the genome and is always inserted into the same site.[8,12,13] The reproducibility of the insertion of LIS-1 and its occurrence during growth make it an excellent marker for classifying a flax genotype as responsive or stable.

To begin to identify the genes controlling these genomic changes, a set of simple sequence repeat (SSR) molecular markers was developed for use in genetic mapping. SSRs, or microsatellites, are tandem repeats of short (2–10 base pair) sequences that often vary in number of repeats, among closely related species and even between varieties within a species, making them useful for genetic mapping.[14] SSR markers have been previously developed in flax from genomic libraries[15–17] and from expressed sequence tag (EST) libraries.[18] Here we report an additional 42 polymorphic SSR

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markers developed from a genomic library enriched for SSRs using a modified fast isolation by amplified fragment length polymorphism of sequences containing repeats (FIASCO) procedure[19] followed by next-generation sequencing, and seven developed informatically. These markers add to the resources available for genetic mapping and variety identification in flax. In addition, many SSRs were polymorphic between stress-responsive and stable flax lines for which reciprocal crosses and F2 generations are available. These SSRs will be used with the F2 offspring grown under stress conditions to construct a genetic map and identify genes involved in the stress response.

Methods

Selection of SSRs from genomic DNA (modified FIASCO procedure)

DNA from the Bison variety of flax, an unresponsive line grown under nonstress conditions, was used for SSR selection. DNA was digested with each of the restriction enzymes HindIII, BamHI (Amersham Life Sciences, Piscataway, NJ, USA), Sau3A (Roche, Indianapolis, IN, USA), HpaII, MspI (New England Biolabs, Ipswich, MA, USA), and Csp6I (Fermentas, Burlington, Ontario, Canada). Digested DNA was cleaned with a Qiaquick polymerase chain reaction (PCR) Purification kit (Quigen, Valencia, CA, USA) according to the manufacturer's instructions, and the purified DNA was dried to a final volume of 20 µl. Each digested DNA was ligated to one 12-mer and one 24-mer adaptor sequence corresponding to the recognition sequence of the enzyme (Table 1) using T4 DNA ligase (Roche, Branchburg, NJ, USA). The ligation reaction was amplified using the respective 24-mer adaptors as previously described.[11] PCR products were hybridized with biotinylated oligonucleotides consisting of repeated sequences to capture SSR sequences in the genomic DNA (CA 15, GTT 7, GACA 5, CAC 7, GAG 7, CA 10, AGG 7, CT 10, AAG 7, TCC 7) as previously described.[19] The products were cleaned using a Qiagen PCR cleanup kit, and sent for 454 sequencing to inqaba biotec (Pretoria, South Africa).

Table 1 Representational difference analysis adaptors and corresponding restriction enzymes

Enzyme	24- and 12-mer adaptors	
BamH1, Sau3A	RBam24	AGC ACT CTC CAG CCT CTC ACC GAG
	RBam12	GAT CCT CGG TGA
HindIII	RBgl24	AGC ACT CTC CAG CCT CTC ACC GCA
	RBgl12	GAT CTG CGG TGA
HpaII, MspI	SHpa24	ACT TCT ACG GCT GAA TTC CGA CAC
	SHpa12	CGG TGT CGG AAT
Csp 6I	RCsp24	AGC ACT CTC CAG CCT CTC ACC GAG
	RCsp12	TAC TCG GTG AGA

Data analysis and primer design

The 454 sequencing data were returned as single-read files and as assembled contigs. The single-read files were reassembled using Bioedit software (version 7.0.0). Single-read and contig data were entered into the program Simple Sequence Repeat Identification Tool (SSRIT; http://www.gramene.org/db/markers/ssrtool) to identify SSRs within the sequences. Sequences containing SSRs with five repeats or more were used to design primers using Primer 3 software (http://frodo.wi.mit.edu/primer3/). There were 103 primer pairs designed, manufactured by MWG Biotech (High Point NC, USA).

Informatic SSR selection

Sequences from clones from the flax variety Stormont Cirrus were aligned with Bethune variety genomic sequence data using BLAST software (http://blast.ncbi.nlm.nih.gov/). Five sequences were found with SSRs that varied in length between the two varieties. In addition, scaffold 218 from the Bethune genome sequence data was analyzed using SSRIT. Ten SSRs were found that contained eight or more tri-nucleotide repeats. Primers were designed using Primer 3 software as described previously and manufactured by MWG Biotech.

PCR

DNA from 12 flax varieties and seven related *Linum* species (Table 2), including the stress-responsive line Stormont Cirrus, were amplified using rTaq DNA polymerase (Takara Bio Inc., Otsu, Japan) with the 103 primer pairs designed from FIASCO selection and the 15 primer pairs designed informatically. PCR involved an initial incubation of 95°C for 1 minute, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, then a final step of 72°C for 5 minutes. PCR products were separated on 3% (w/v) agarose gels in 0.5 X TBE (1 X TBE = 100 mM Tris, 90 mM boric acid, 1 mM ethylenediaminetetraacetic acid, pH 8.4).

PIC value and allele number

PIC values were calculated using the standard formula:[20]

$$PIC_i = 1 - \sum_{j=1}^{k} P_{ij}^2 \quad (1)$$

Table 2 Flax varieties and species used in polymerase chain reaction

Variety	Origin
Stormont Cirrus	From A. Durrant
Stormont Motley	USDA
CDC Bethune	University of Saskatoon
CI1303	C Dean Dybing, North Dakota
Koto	USDA
AB	USDA
Bolley Golden Sel	USDA
Burke	USDA
Lira Prince	USDA
Hollandia	USDA
Rembrandt	USDA
Royal	USDA
L. grandiflorum	Portugal
L. strictum	Portugal
L. perenne	Aachen
L. narbonense	Chelsea
L. grandiflorum coeruleum	Romania
L. bienne	Aachen
L. austriacum	Romania

Abbreviation: USDA, US Department of Agriculture

where P_{ij} is the frequency of the *j*th allele for the *i*th marker within the set of flax species and varieties tested, and summation extends over *k* alleles detected for the *i*th marker.

The number of alleles was determined by counting every size class of bands that amplified across all varieties and species tested for any given marker.[18]

Results

Sequencing of the selected genomic DNA returned 13,677 reads with a total of 2,440,703 bases. These were assembled into 1503 contigs (containing more than one read) containing 186,197 bases, which constituted 0.027% of the total flax genome, which is estimated to be 700 Mb.[21]

Analysis of the contigs with SSRIT showed that 245 contigs contained SSRs with five or more repeats. Primer pairs were made from 62 contigs that had enough sequence surrounding the SSR from which to design primers. Single-read sequences were also analyzed with SSRIT, and 41 primer pairs were made from sequences not found in the contigs and containing five or more repeats. Of 103 primer pairs tested, 11 gave no amplification. Of the 92 primer pairs that amplified, 45 were monomorphic in the flax varieties and *Linum* species that were tested, and five pairs gave patterns of many bands of sizes not consistent with being due to differences in the number of SSR repeats and so were not used as markers in this study. Of the 42 primer pairs (46%) that were polymorphic, the number of alleles ranged from two to eight, with an average of 3.32. PIC values ranged from 0.1049 to 0.8642, with an average of 0.47 (Table 3).

When comparisons were made using only the 12 flax varieties, the SSRs were less polymorphic. Nine additional primer pairs were monomorphic within the varieties, lowering the percent of polymorphic primers to 36%. The average number of alleles among varieties was 3.09, and the average PIC was 0.46.

For the informatically derived SSRs, seven out of 15 (47%) were polymorphic among varieties and species, the average number of alleles was 4.14, and the average PIC was 0.5872. Among varieties only, the same percentage polymorphism (47%) held, the average number of alleles was 2.86, and average PIC was 0.5022.

Polymorphism of SSR sequences fall within the range of those for previously reported genomic and EST derived SSRs.[15–18] All of these polymorphic SSR sequences were registered in GenBank (www.ncbi.nlm.nih.gov/genbank). Accession numbers are listed in Table 3.

Discussion

These data add to the growing number of SSR primers available for flax for use in mapping, variety identification, and phylogenetics. Other markers available for flax include RFLPs (restriction fragment length polymorphisms),[22] RAPDs (random amplified polymorphic DNA),[22–26] AFLPs,[27–30] and SSRs.[15–18]

Informatically derived SSRs were more polymorphic than genomic SSRs found using FIASCO, but this is not surprising considering that many of the informatically derived SSRs were chosen because of previously known polymorphisms between two closely related flax varieties. We expected to find a higher PIC in genomic SSRs compared with those from EST libraries[18] because of the lack of constraint on the number of repeats in noncoding regions of the genome, but this was not the case. The percentage of markers that were polymorphic among flax varieties was lower in genomic SSRs (36% in genomic, 40% in EST), and the average PIC was not significantly different between the two sets (0.46 ± 0.16 genomic, 0.35 ± 0.17 ESTs, Student's *t*-test, $P > 0.1$). EST values are reported for flax varieties only, so no comparison is possible for values when related *Linum* species are screened. The PICs of other genomic SSRs reported were 0.601,[15] 0.33,[16] and 0. 542.[17] These genomic SSRs are a valuable resource because they are dispersed throughout the genome, rather than clustered in gene-dense regions. When used for mapping, the wider distribution of genomic SSRs makes them an ideal complement to the EST SSRs, which have the advantage of directly tagging the genes they reside in.[18] Together, these resources can generate a map that is spread evenly over the genome, with the relative locations of many genes being tagged. SSRs that

Table 3 Sequence, TM, GC content, length, motif, number of repeats, number of alleles, and PIC values for polymorphic SSR primer pairs

Primer name	Sequence (5'→3')	TM (°C)	GC content (%)	Length (bp)	Motif	Number of repeats	Number of alleles	PIC	GenBank accession
45L	GCCTAAAGCTGATGCGTTTC	57.3	50	20	TCT	11	4	0.519	HQ230810
45R	GGCTCCTTCTTATGCAAGACA	57.9	47.6	21					
101L	AGGAAGAGGTAGCCCAGTCC	61.4	60	20	GAA	7	3	0.406	HQ230811
101R	AGACTCACGGTGAAGGCAAC	59.4	55	20					
258L	TGGGGTATACACGAGTCAGGA	59.8	52.4	21	GAA	7	4	0.627	HQ230812
258R	CGCACTCGACAAGGACTACC	61.4	60	20					
334L	CGGGAAAGTTACGGAGAGC	58.8	57.9	19	AAG	7	3	0.531	HQ230813
334R	TTCCTCCTCCTCACAACCAC	59.4	55	20					
681L	GGTTTACTCCCCCAGAGGTC	61.4	60	20	TTC	7	3	0.292	HQ230814
681R	GGAAACGCTAAATTGGCAGA	55.2	45	20					
900L	TGGGATTCAAGTATTAAATGCAG	55.3	34.8	23	GAA	12	2	0.231	HQ230815
900R	TCCAGTAGTTCTTCTTGCTCCA	58.4	45.5	22					
912L	CCGGCTGACTACGATTTGTT	57.3	50	20	TCT	9	4	0.593	HQ230816
912R	ATACCCTAACCCTGCTGCAA	57.3	50	20					
947L	TGCAACAACAACAACCGTTA	53.2	40	20	AAG	7	3	0.598	HQ230817
947R	ACCGCCAGCTCAGGATAAT	56.7	52.6	19					
959L	TCCCAACAACAATAACCAACC	55.9	42.9	21	ATG	7	3	0.576	HQ230818
959R	CCACCTGCACCAACACTAAA	57.3	50	20					
963L	GACGAGGACGAGGAAGAAGA	59.4	55	20	GAG	7	3	0.463	HQ230819
963R	CCTCCCAAGTTATAACCTATCCTG	61	45.8	24					
1019L	AATAGTGGGGAAAGGCTTGG	57.3	50	20	TTC	7	2	0.105	HQ230820
1019R	GCGCGATTACTGCTTTGAAT	55.2	45	20					
10 L	TTGAAGACATGCAACCCATC	58.35	45	20	CTT	5	2	0.198	HQ230821
10 R	TTTGACAGACACCAACATCG	58.35	45	20					
14 L	TTTGACAGACACCAACATCG	58.35	45	20	GGT	5	2	0.198	HQ230822
14 R	GACACCGGACACACCAAATC	62.45	55	20					
30 L	GTTTCCGCTGCCGATACTCT	62.45	55	20	TTC	5	2	0.198	HQ230823
30 R	AAAATTAGGCGGCGAAGAAG	58.35	45	20					
31 L	ACGGCGAAGACGAGGATAA	60.16	52.6	19	CTC	5	3	0.370	HQ230824
31 R	GGGGTTCGTCGTTTTCTTCT	60.4	50	20	GAC	5			
33 L	CGCTCCGAATAATGACCATA	58.35	45	20	TCT	5	2	0.198	HQ230825
33 R	GCAAAAGTGGGAACAGAGGA	60.4	50	20					
35 L	ACGTCGAGGAGAAGGGAGAT	62.45	55	20	GAG	6	3	0.494	HQ230826
35 R	AATGTCCGTCTCCCACAAAC	60.4	50	20					
41 L	TGAAGAAACTGGACAAACTTCAA	57.42	34.8	23	TTC	5	2	0.198	HQ230827
41 R	TGATGTTGGGGAGGATAGGA	60.4	50	20					
50 L	TTTGCTTTGCAATTCACTCG	56.3	40	20	AGA	6	2	0.198	HQ230828
50 R	GCAACAGAAGCAACTCGACA	60.4	50	20					
64 L	ATGAAGACCGGTGCTGTTG	60.16	52.6	19	GGT	5	6	0.741	HQ230829
64 R	GTCCAGCCAGTCAGTTCGAT	62.45	55	20	GCT	5			

86 L	CTGGAGCATGGACAGAGAAA	60.4	50	20	TTC	6	2	0.198	HQ230830
86 R	GCAGGATTCACAACCGAAAG	60.4	50	20					
96 L	ACCGTCATCTCCTCGACAAC	62.45	55	20	GCC	5	3	0.370	HQ230831
96 R	CTGGACGCTTTTGTTTGGTG	60.4	50	20					
108 L	TCCATCTGGAAGCAGGATAA	58.35	45	20	GAG	5	3	0.568	HQ248104
108 R	CTGGGATCCTCCCAAGAGA	62.32	57.9	19					
128 L	TGAACTGTCACGACCAGCAT	60.4	50	20	CCA	5	6	0.741	HQ230832
128 R	CTACGGCTGAATTCCGACAC	62.45	55	20					
134 L	TGATGGCTGTTAAGGAGAGGA	60.61	47.6	21	AGA	5	4	0.617	HQ230833
134 R	TCACACAACATGTCCACCTTC	60.61	47.6	21					
SR0355 L	TGGATTAGCTAGCTCCAACTTTC	60.99	43.5	23	TCT	11	3	0.642	HQ230834
SR0355 R	CGTCGTTAGCTAGAGGGTTGA	62.57	52.4	21					
SR1551 L	GGCTGCTGCTGATCTTGTT	60.16	52.6	19	TCT	5	8	0.864	HQ230835
SR1551 R	CTGCGACACTAGCACTCCAA	62.45	55	20	TCA	10			
SR1039 L	CATTCTGAGGATTTGCTCTGG	60.61	47.6	21	TTC	10	2	0.375	HQ230836
SR1039 R	TGACCGTCATGAGCTTTTCTT	58.66	42.9	21					
SR1753 L	TTCCTTTTCGCAACTTCACA	56.3	40	20	AGAC	5	2	0.219	HQ230837
SR1753 R	CTTCCCTTTGCTTCCTCTGA	60.4	50	20					
SR1757 L	AGACGCACTCCACCTGAC	62.18	61.1	18	CAA	19	7	0.857	HQ230838
SR1757 R	AGTTCAACTCGTGGTGGTTG	60.4	50	20					
SR2395 L	CCGCAATGTCTCCTTCAAAA	58.35	45	20	TTC	10	3	0.406	HQ230839
SR2395 R	ATGAGAATCGGGGGTAAAGG	60.4	50	20					
SR2575 L	TCTCCCATCTCCTTGTCCAC	62.45	55	20	CTT	10	2	0.444	HQ230840
SR2575 R	TTACCCTCGCAATGAATGTG	58.35	45	20					
SR2368 L	TTCGTCTCACCCATGTAAGTTTT	59.2	39.1	23	TCT	12	2	0.198	HQ230841
SR2368 R	CCCAAGAAAACCGGAGAAAT	58.35	45	20					
SR1496 L	TTGTCGTCTCTCCGCTTCTT	60.4	50	20	TCT	11	2	0.444	HQ230842
SR1496 R	GACGGAGTCATGCCCTAAAC	62.45	55	20					
SR1468 L	TCCATCACCTTCCTCATCCT	60.4	50	20	TCT	13	2	0.494	HQ230843
SR1468 R	TTGGGGAGTTCAAGATTAAATAGC	59.44	37.5	24					
SR2862 L	CAGGCTCCATGGTGGATTA	60.16	52.6	19	TTC	16	7	0.815	HQ230844
SR2862 R	AGCCAGGTGTCCCGCCAT	64.46	66.7	18					
SR0885 L	CTGCCGTCTTCCTCATCAAT	60.4	50	20	TCT	10	4	0.716	HQ230845
SR0885 R	GTAGGCGGCTGGTGATGAT	62.32	57.9	19					
SR3372 L	GGACAGGAACTCCATTGACG	62.45	55	20	TTC	11	4	0.716	HQ230846
SR3372 R	CTACCCAGACCCCAAACTCA	62.45	55	20					
SR0151 L	CCTTTGACTTCCTTCCACCA	60.4	50	20	TTC	11	2	0.219	HQ230847
SR0151 R	GAAATCGAAACAACCGAAACA	56.71	38.1	21					
SR1894 L	GGGTTGATGCTTGAACTTGA	58.35	45	20	TCT	12	6	0.815	HQ230848
SR1894 R	GCACCTTCGTCAATACCTCCT	62.57	52.4	21	TTC	9			
SR2240 L	CCCCCTTATCGCTCTGTAAA	60.4	50	20	TCT	12	2	0.346	HQ230849
SR2240 R	TTTGGTTGCGTTGGTGATAA	56.3	40	20					

(*Continued*)

Table 3 (*Continued*)

Primer name	Sequence (5'→3')	TM (°C)	GC content (%)	Length (bp)	Motif	Number of repeats	Number of alleles	PIC	GenBank accession
SR3572 L	TCTCGTAGCTAGGGAGATGG	62.45	55	20	GTC	10	4	0.667	HQ230850
SR3572 R	AAAGCCGTCGTACTCACCAC	62.45	55	20					
JA 23 L	TCTGTCTCCAACCTCGGCCA	64.5	60	20	NA	NA	5	0.577	HQ259246
JA 23 R	TCAAACCGAAGCAAACAGTG	58.4	45	20					
C 31 L	GCCCTGAGCTCATTCACCTA	55	62.4	20	ACAA	4	3	0.561	HQ259245
C 31 R	GAATTGTGGAACGGGATGAA	58.4	45	20					
33096 L	AATTACTCCAGGAATCAACAAAAA	56	29.2	24	TCG	8	3	0.615	HQ259247
33096 R	AAAGAGAGAAGCCAGCAACAA	58.7	42.9	21					
85726 L	AGAAATCTGGTACGAGAACATCA	59.2	39.1	23	GAT	9	5	0.714	HQ259248
85726 R	TCGCTATCCGCTTCTACACC	62.4	55	20					
188103 L	GAAAGACTCGGGTGTCTCGT	62.4	55	20	TTC	114	7	0.786	HQ259249
188103 R	CTTGCCAAGCGTGCAATTAT	58.4	45	20					
506187 L	CTCCCACGCCCAATTATTC	60.2	52.6	19	GGA	8	2	0.301	HQ259250
506187 R	GTGGAGCGATTTTCTGATCC	60.4	50	20					
668637 L	AACCAGTCATCCGTGACCTC	62.4	55	20	ATC	8	4	0.556	HQ259251
668637 R	TTGTGAGAGAGTTTGTGGGTTT	58.9	40.9	22					

Abbreviations: TM, temperature; PIC, polymorphism information content; GC, guanine–cytosine; SSR, simple sequence repeat; bp, base pair.

are polymorphic between the cultivars Bethune (a stable cultivar) and Stormont cirrus (a responsive cultivar) will be used to create a genetic map of the flax genome, which will enable the identification of the loci controlling the ability of flax varieties to respond to environmental growth conditions by modifying their genome.[8]

Disclosure

The authors report no conflicts of interest in this work.

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