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Bulk Segregant Analysis Technique to Target Seasonality and Runnering Loci in Diploid Strawberries

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Abstract - Bulked segregant analysis was used to determine inter-simple sequence repeat DNA (ISSR) markers for two genes, one controls seasonality and other runnering in diploid *Fragaria spp*. ISSR- PCR analysis was performed using these DNA pools as template. The bulks are screened for differences using anchored simple sequence polymorphic DNA primers which generates clean band profiles and polymorphic fragments were detected. Using 23 primers, either singly or pairwise, we established 16 markers tightly linked to the seasonality locus and 7 markers for runnering locus. This bulked segregant method can be applied for any species and any region of interest in which detailed linkage maps or physical maps are needed.

Keywords -
Strawberry, ISSR, Bulk, Marker.

1. Inroduction

Fragaria vesca L.(syn. *Fragaria silvestris* Duch.; *Fragaria vulgaris* Ehr.) is a wild species. It played an important role in the fruit garden for several centuries as a predecessor of cultivated strawberries **[1].** The European 'Alpine' strawberries, *Fragaria vesca semperflorens*, are naturally-occuring everbearing forms of *Fragaria vesca* but are diploid (2n=14) like *F. vesca*. Hiirsalmi **[2]** described *F.vesca semperflorens* as a continuously fruiting mutant of *F. vesca* under the name Minja. Although wild type *F. vesca* produces runners and has a seasonal flowering habit, the alpine cultivars produce no runners. *Fragaria vesca* shows two fairly distinct phases of development. After the plants have finished flowering in spring they enter a vegetative growth phase which continues until autumn when flower primordia are initiated; these then grow out and produce fruit in early or late summer. The distinct everbearing and seasonal fruiting forms of *F. vesca* differ by a single gene allele, the recessive being everbearing **[3].** The dominant allele of the seasonality gene is believed to be inactivated by cool temperatures and short day (SD) in the autumn **[4].** The small number of chromosomes (2n=14), short generation time (4-5 months) and small genome size (0.168pg), means that *Fragaria vesca* has potential as a model species to improve our understanding of flowering in perennials. Additionally, because plants of *F. vesca* are small they are well suited to manipulation of flowering in environmentally controlled growth cabinets.

Bulk Segregant Analysis; Several methods have been described to detect linkage of molecular markers to a target gene. The most widely applied of these uses two subsets of individuals from a segregating population that share the same allele for the target gene.

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This method is often restricted to segregating generations which are simplest and cheapest to produce, such as Backcross (Bc) and F_2 generations. Wang and Paterson **[5]** concluded that of these two generations, the Bc would generally be the best with which to work. Markers are sought that are present in most individuals of one pool but are largely absent from those of another.

Pools of DNA from different sub-populations can be used as templates of DNA to screen for the presence of pool-specific markers **[6].** The feasibility of using a pooled-DNA approach for dominant markers was tested using individuals from a *Lactuca sativa* population segregating for turnip mosaic potyvirus resistance **[7].** Durham and Korban **[8]** identified polymorphic DNA markers, based on the presence or absence of bands, among bulked apple cultivars using a set of 59 oligonucleotide decamer primers. Of 80 random sequence primers examined, 3 amplified products were found only in the profile from a *B. indicus* DNA pool **[9]**. Two RAPD markers linked to the gene block of interest were identified by using pooled DNA samples of genotyped individuals from two segregating populations **[10]**. There appears to be no consensus for the number of samples contained pool used for this purpose. The numbers employed in previous works are highly variable; 5 by Naqvi *et al.* **[11]**, 10-15 by Gavora *et al.* **[12]**, 16 by Wain *et al.* **[13]**, 11 and 22 by Gallego *et al.* **[14]**.

Inter- Simple Sequence Repeat (ISSR); This system employs single primers that contain sequences that are complementary to simple sequence repeats but also include a 1-4 base 'anchor' at 3' or 5' termini **[15]**. The use of two ISSR primers in combination, however, creates additional scope for PCR artifacts **[16]**. The anchor can contain any base combination other than that which continues the repeat motif and serves to ensure that the primers bind to 3' or 5' end of the SSR template. PCR generates many products that largely comprise of DNA sequence between neighbouring inverted SSRs. The complex products amplified are then fractionated on polyacrylamide gels for pattern visualization. These amplified bands are mostly dominant markers and can be used in wide range of plant species. This technique permits detection of polymorphism in microsatellite and inter microsatellite loci without previous knowledge of DNA sequences **[15; 17].** Comparison of other methods i.e., RAPD, by using the same DNA, equipment and segregation and detection technique, i.e., agarose and ethidium bromide, indicated that ISSR gave a better band reproducibility **[18]**. Slight changes at the anchor can produce different band patterns **[19].**

The objectives of the work presented here to generate molecular markers linked to seasonality and runnering loci in diploid strawberry by bulk segregant method using Back cross (Bc) population and to determine the relative advantages of BSA for quantitative traits with ISSRs, a dominant marker system.

2. Material and Method

Plant material; Two forms of the diploid strawberry were used: one exhibits seasonal flowering and produces runners (*F. vesca*), the other flowers continually and has a lack of runners (*F. vesca semperflorens*). In other respects, these forms are morphologically indistinguishable and genetically very similar. Following a cross between seasonal *F. vesca* and everbearing *F. vesca semperflorens*, the F1s were backcrossed with the everbearing parent to deduce those markers showing genetic linkage with the seasonality and runnering traits. This was performed using bulk DNA samples of plants exhibiting the same phenotype (i.e. seasonal runnering etc) from BC1 segregating progenies.

Genomic DNA extraction and ISSR-PCR analysis; Genomic DNA was isolated from unexpanded leaves of both parental lines and the F_1 hybrid using the method described by Doyle and Doyle **[20]**. Extracted DNA was quantified using a DyNA 200 fluorimeter (Amersham Pharmacia Biotech) according to the manufacturer's instructions.
ISSR-PCR analysis was performed according to the protocol described by Charters *et al.* **[21]** using one or a combination of two primers. 10ng of genomic DNA was added to a reaction mixture containing 1 unit *Taq* polymerase, 5mM MgCl buffer, 0.2 mM of each dNTP (all Boehringher Mannheim) and 0.2 μM of primers. DNA was amplified on a Hybaid Omnigene thermocycler using the following programme: 1 min at 94 ^{o}C and 30 cycles of (1 min at 94 ^{o}C 2 min at 55 ^{o}C and 30 sec at 72 ^{o}C) followed by final 5 min extension at 72 ^{o}C. Amplification products were loaded onto precast polyacrylamide gel comprising a 5% w/v acrylamide stacking gel and 10% w/v acrylamide resolving gel (Amersham Pharmacia Biotech, Celangel 48S). They were then fractionated and silver stained according to the procedure described by Charters *et al.* **[21]**.

Marker generation and selection; A backcross programme was needed to allow introgession, mapping and ultimately the isolation of the seasonal flowering gene from *Fragaria vesca*. The initial aim was to identify useful ISSR-PCR markers that are only present in the band profile of the seasonal flowering *Fragaria vesca* since both seasonality and runnering are dominant.

Pooling of DNA; ISSR analysis was used for the rapid identification of DNA markers linked to the seasonality and runnering loci of *F. vesca* genome. Fifty BC_1 plants representing all four possible phenotypes (Seasonal runnering, Seasonal non-runnering, Perpetual runnering, Perpetual non-runnering), derived from a *F. vesca* x *F.v. semperflorens* cross, were randomly chosen for analysis. DNA was extracted and quantified as above, and 100 ng of DNA from each individual of the same group were then pooled, resulting in a total of 150 μl (a concentration of 33.3 ng/μl) of DNA stock solution. Those four stocks of pooled DNA were used as templates for ISSR PCR using 23 single primers and 45 primer pair combinations to generate candidate markers.

3. Results and Discussions

The value of ISSR-PCR for generating clear band profiles for *F. vesca*
There were 100 UBC ISSR primers tested on the parental *F. vesca* and *F.vesca semperflorens* genomes. The value of each primer for subsequent analysis was assessed according to the number and distinctiveness of diagnostic bands generated. The primers scored as unsuccessful failed to produce band profiles in either or both sub-species or else failed to generate any clean scorable profiles. In total, 23 out of 100 primers were found to be useful in this respect. The selected primers were then screened for the presence of molecular markers associated with the seasonal flowering and runnering traits in *F. vesca*. The total number of clearly scorable bands produced by each of the 23 selected primers varied from 3 (primer 821) to 16 (primer 888).

Single primers
The 23 primers identified above were screened to compare the band profiles of the two parent species, and the F_1 hybrid. Markers were selected that were present only in the PCR products from *F. vesca* and the F_1 hybrid. Relatively few polymorphisms were observed between parents, and some primers failed to reveal any polymorphisms. Non-polymorphic primers were, then, not used for the further analysis**.** Primer 888 produced the greatest

variation in banding profile between the two parents. In total, 16 polymorphic band were obtained from 14 primers (807, 810, 811, 826, 835, 841, 842, 844, 856, 881, 888, 889, 890, 891). The remaining 9 out of 23 primers failed to generate any polymorphism. Some of the plymorphic bands were spefic

Use of primer pairs in ISSR PCR

An experiment carried out with all 45 primer pair combinations using 10 out of 14 primers showed that the ISSR-PCR with primer pairs could produce additional low molecular weight markers (Table 1). When two primers were used together in a PCR, not only were the banding profiles produced by single primers combined, but novel bands were also produced. Figure 1 B shows some PCR products in which four seasonal spesific markers obtained from three primer combinations. Any of these primer had not been appeared when the primers used as singles.

Table 1. UBC microsatellite primers giving informative markers, their repeating sequences and Tm for PCR

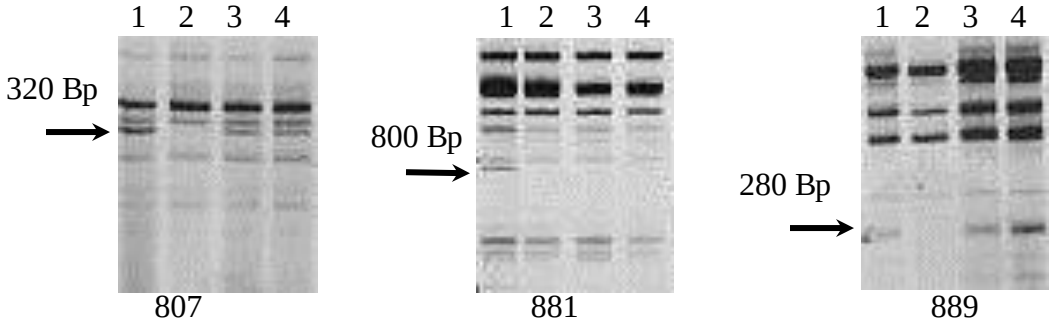
Primer	Sequence	Repeat	Tm for PCR
807	AGA GAG AGA GAG AGA GT	(AG)8 T	50
810	GAG AGA GAG AGA GAG AT	(GA)8 T	50
811	GAG AGA GAG AGA GAG AC	(GA)8 C	52
826	ACA CAC ACA CAC ACA CC	(AC)8 C	52
835	AGA GAG AGA GAG AGA GYC	(AG)8 YC	56/54
841	GAG AGA GAG AGA GAG AYC	(GA)8 YC	54/56
842	GAG AGA GAG AGA GAG AYG	(GA)8 YG	54/56
844	CTC TCT CTC TCT CTC TRC	(CT)8 RC	52/54
856	ACA CAC ACA CAC ACA CYA	(AC)8 YA	52/54
881	GGG TGG GTG GGT GGG T	(GGGT)4	54
888	BDB CAC ACA CAC ACA CA	BDB(CA)7	app. 48-52
889	DBD ACA CAC ACA CAC AC	DBD(AC)7	app. 48-52
890	VHV GTG TGT GTG TGT GT	VHV(GT)7	app. 48-52
891	HVH TGT GTG TGT GTG TG	HVH(TG)7	app. 48-52

N= (A, G, C, T) R= (A, G) Y= (C, T) Tm= (4 X GC) + (2 X AT)
B= (C, G, T) (i. e. not A) D= (A, G, T) (i. e. not C)
H= (A, C, T) (i. e. not G) V= (A, C, G) (i. e. not T)

Markers generated in bulk DNA

Bulked segregant analysis is based on the premise that when DNA samples are bulked, polymorphic markers that are common to individuals within the bulked sample, but absent among individuals of other bulked samples, will appear polymorphic when bulked samples are compared. On the other hand, polymorphic markers that segregate at random among the individuals that comprise two bulked samples will fail to show polymorphisms when the bulked samples are compared.

A.



B.

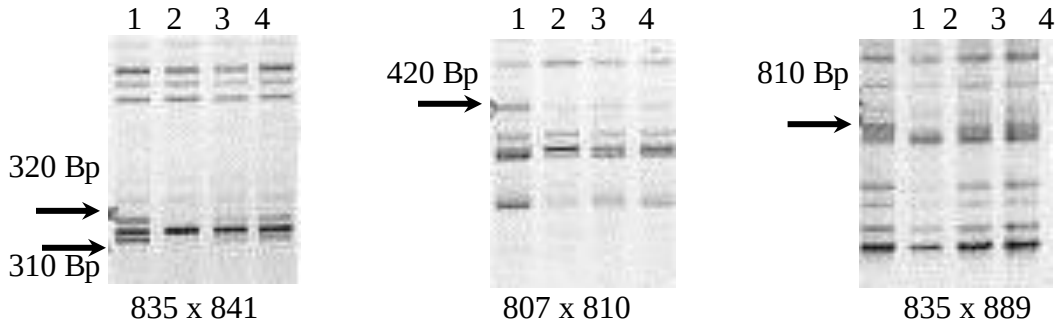


Figure 1. ISSR markers linked to seasonality locus, generated by **A)** single primer, **B)** primer pairs using BC_1 segragation bulks. *arrows* indicate *Fragaria vesca f. vesca* alleles in polymorphic loci, *Lane 1* Seasonal-Runnering, *Lane 2*: Seasonal-Nonrunnering, *Lane 3*: Perpetual-Runnering, *Lane 4*: Perperual-Nonrunnering)

The ISSR primers and primer pairs identified in the preliminary experiment were used to screen for the differences between two pairs of bulked BC1 progenies: the seasonal-perpetual pair (for seasonality locus) and the runnering –nonrunnering pair (for the runnering locus). The generated markers from some of single primers were illustrated in Figure 1A.The informative markers generated by 807, 841 and 888 were strong and distinct but markers from the other primers were primarily minor bands. Four out of 23 single primers used yielded markers that were polymorphic between two pools for the seasonality locus, giving (indicated by primer followed by size in bp in subscript) 807300, 856850, 856700, 889300, 889150 and 890300 were present in seasonal bulk but absent in the perpetual bulk.

No marker was obtained for the runnering locus using single primers. The use of two ISSR primers in combination (45 combinations) yielded 12 markers for the seasonality locus and 7 markers for runnering locus.

The usefulness of ISSR primers for the *F. vesca* genome

One advantage of ISSR-PCR primers over a system such as SSR-PCR is that no prior knowledge of the genome is required. The primers are designed around one of a number of core repeat motif sequences. In the present study, the appearance of complex profiles only using primers designed to bind to dinucleotide repeats suggests that two base repeats are common in the *F. vesca* genome and by application of trinucleodite and tetra nucleodite repeats are less abundant. The position of the anchor was also important in designing the complexity of profile generated. Primers anchored at the 5’ termini generated more bands than those anchored 3’ end. This is probably attributable to the direction of extension from the primer. *Taq* polymerase is less able to extend when primer-template mismatches occur at the 3' end than at the 5' end. Thus, if a mismatch accurs in a 3' anchor amplification by PCR will be less likely than if the mismatch accurs in a 5' anchor. Changes to the length of the anchor appeared also to be important in determining the number of bands generated. Primers with short (1 or 2 anchors) or including some degeneracy tended to produce more bands than those with larger anchor. The primers resulting in amplicon were mostly three prime anchoreds. The results also confirm the ability of primers, based on SSR motifs, to produce fragments that are useful as genetic markers.

Polymorphism

The ISSR-PCR markers showed virtually complete agreement with expectations of Mendelian segregation, for a dominantly inherited system. This predictability is important for its use in genetic linkage analysis. The Mendelian inheritance of the ISSR markers also indicated that *F. vesca* and *F. v. semperflorens* are relatively unbiased and showed low heterozygosity. These two forms would thus appear to constitute a suitable population for mapping of seasonal flowering and runnering genes. Although there is little polymorphism found between these species the degree of polymorphism detected in the ISSR-PCR studies is comparatively higher than using AFLP markers (Moreno *et al.*, 1998). Given the close genetic similarity between *F. vesca* and *F.v. semperflorens*, the probability of finding a marker within a specified distance of a target gene depends on the genome, the sequence divergence in the region scanning the target gene and the ability of ISSR primers to reveal these polymorphisms.

Bulked DNA analysis

BSA (Bulked Segregant Analysis) is an efficient procedure to detect markers linked to target loci. The success of the approach depends on the genetic divergence between the parents in the target region. The parental genomes used in this work apparently few characters and also the use of bulked analysis, provided a means of screening large numbers of potential markers to identify those linked to the target trait.

The minimum size of bulk can be determined by the type of marker being screened (dominant or codominant), and the size and the type of population used to generate bulks (F2, backcross, etc.) **[6]**. Pooling a large number of individuals increases the probability that two pools may not differ for alleles other than target loci. However, as the pool size increases, so does the probability that individuals may occur in the pool with a double crossover within the interval. Decreasing the number in the pool, on the other hand, may result in increased numbers of false positives during individual screening **[7]**.

4. Conclusion

The BSA technique can be used to target any important locus. The method can reduce cost by several-fold, particularly when used with PCR-based techniques. In this study, 18 candidate markers were identified as putatively linked to the seasonality locus and 7 as possibly linked to the runnering gene. These markers were generated in a short time by screening 68 primers and primer combinations using BSA. The markers generated from pooled progenies could then be screened on individual plants to produce a local map around seasonality locus.

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