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ORIGINAL PAPER

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The development of ISSR-derived SCAR markers around the *SEASONAL FLOWERING LOCUS* (*SFL*) in *Fragaria vesca*

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Abstract *Fragaria vesca* is a short-lived perennial with a seasonal-flowering habit. Seasonality of flowering is widespread in the *Rosaceae* and is also found in the majority of temperate polycarpic perennials. Genetic analysis has shown that seasonal flowering is controlled by a single gene in *F. vesca*, the *SEASONAL FLOWERING LOCUS* (*SFL*). Here, we report progress towards the marker-assisted selection and positional cloning of *SFL*, in which three ISSR markers linked to *SFL* were converted to locus-specific sequence-characterized amplified region (SCAR1–SCAR3) markers to allow large-scale screening of mapping progenies. We believe this is the first study describing the development of SCAR markers from ISSR profiles. The work also provides useful insight into the nature of polymorphisms generated by the ISSR marker system. Our results indicate that the ISSR polymorphisms originally detected were probably caused by point mutations in the positions targeted by primer anchors (causing differential PCR failure), by indels within the amplicon (leading to variation in amplicon size) and by internal sequence differences (leading to variation in DNA folding and so in band mobility). The cause of the original ISSR polymorphism was important in the selection of appropriate strategies for SCAR-marker development. The SCAR markers produced were mapped using a *F. vesca* f. *vesca* × *F. vesca* f. *semperflorens* testcross population. Marker SCAR2 was inseparable from the *SFL*, whereas SCAR1 mapped 3.0 cM to the north of the gene and SCAR3 1.7 cM to its south.

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Introduction

The *SEASONAL FLOWERING LOCUS* (*SFL*) gene regulates seasonal flowering in the wild diploid strawberry, *Fragaria vesca.* Plants carrying the dominant allele of the *SFL* gene exhibit a restricted-flowering period (typically late April to early June), whereas genotypes that are homozygous for the recessive allele flower almost continuously throughout the year (Brown and Wareing 1965; Albani et al. 2001). *SFL* thereby controls a character of potentially major adaptive significance in the life cycle of the short-lived perennial *F. vesca*. Concentration of the flowering effort for a short period not only enhances the chances of outcrossing (Barrett et al. 1996) but also fundamentally alters the relative allocation of resources between reproductive and vegetative growth (Albani et al. 2001). The phenology of the seasonal-flowering habit has been described among other temperate perennials. However, the molecular mechanisms underlying this phenomenon are poorly explored. This is generally attributed to problems associated with the biology of perennials (e.g. prolonged juvenility and large size) and often also to their complex genetics.

F. vesca is a diploid perennial and its small genome size (164 Mb) (Akiyama et al. 2001) makes it a useful model for genetic studies. More importantly, the inheritance of the seasonal-flowering character in F_2 and backcross progenies from crosses between the seasonal-flowering *F. vesca* f. *vesca* and the perpetual-flowering *F. vesca* f. *semperflorens* matches that of a single Mendelian gene, the *SFL* (Brown and Wareing 1965; Cekic et al. 2001). This is in contrast to the situation in the cultivated strawberry (*F.* x *ananassa*), where the perpetual-flowering character appears to be the dominant trait but is apparently controlled by several genes (Battey et al. 1998). Isolation of *SFL* would clearly provide insight into the seasonal control of flowering in diploid *F. vesca*, but may also ultimately reveal (by complementation studies) whether *SFL* plays any role in flowering in the polyploid *Fragaria* species into which *F. vesca* has contributed genomes. Development of molecular markers closely linked to the

SFL gene represents the first step towards its isolation by positional cloning. We have previously reported that combination of different ISSR primers can generate markers linked to *SFL* and have potential value for both purposes (Cekic et al. 2001). Here, we screened additional ISSR primer combinations using bulked segregant analysis (BSA) to identify more *SFL*-linked markers.

Amplification by PCR using pairs of ISSR primers that target different SSR motifs can generate additional polymorphism but also results in a much more complex profile (Cekic et al. 2001). The complexity of data scoring restricts the utility of methods of this kind for marker-assisted breeding and positional-cloning applications. For this reason, an increasing number of researchers have adopted a strategy of converting markers identified in multilocus marker systems (e.g. RAPDs, ISSRs and AFLPs) into locus-specific markers. The ease with which a targeted product can be converted into a simple, locus-specific marker varies according to the technique used to produce the original multilocus profile. Such marker conversion has been most frequently described from RAPD band profiles (Tartarini et al. 1999; Brisse et al. 2000; Cao et al. 2001). Fewer groups have reported the production of locus-specific markers from AFLP profiles (Behura et al. 2000; Negi et al. 2000), and to our knowledge, this is the first attempt at marker conversion from ISSR-PCR profiles. The ISSR marker system usually generates a less complex profile than AFLP but can yield polymorphisms between profiles in many ways. In this study, we characterise and then exploit three different causes of ISSR band-profile polymorphism to produce contrasting forms of locus-specific markers that are closely linked to the *SFL*.

Materials and methods

Plant material

Plant material of the seasonal-flowering parent *Fragaria vesca* f. *vesca* (*SFL*/*SFL*) was obtained from Dr. D.W. Simpson, Horticulture Research International, East Malling, UK. The perpetually-flowering parent *F. vesca* f. *semperflorens* (*sfl*/*sfl*) was kindly provided from the personal breeding collection of Dr. J.K. Jones, The University of Reading, UK. A BC$_1$progeny generated from hybridisation of these lines comprised 1,049 individuals. Flowering characteristics were scored for plants in this progeny when grown in an unheated glasshouse between January 1999 and December 2000 (Albani et al. 2001). Plants exhibiting a perpetual phenotype (genotype *sfl*/*sfl*) flowered strongly throughout the growing season. Plants deemed to be seasonal (genotype *SFL*/*sfl*) typically flowered between March and August. A randomly selected subset of 100 plants exhibiting seasonal flowering, and 100 perpetually flowering plants were taken from the progeny and used in the development of sequence-characterized amplified region (SCAR) markers below. Mapping of the SCAR markers was performed on the entire progeny of 1,049 plants.

DNA isolation

For the genetic mapping of the *SFL*, DNA of the parental clones and the BC$_1$ progeny was extracted using the method described by Doyle and Doyle (1987). Parental DNA used for the development of the locus-specific markers was extracted with the DNeasy plant mini kit (Qiagen) according to the manufacturer's instructions.

Bulked segregant analysis

Equal quantities of DNA from 100 seasonally flowering BC$_1$ plants and from 100 perpetually flowering plants were each mixed to produce the 'seasonal-flowering (S)' and 'perpetual-flowering (P)' pools, respectively. The S and P pools were used as template DNA to screen 23 ISSR primer combinations (Table 1) according to the ISSR protocol described by Charters et al. (1996). Candidate markers generated by the BSA were screened to confirm linkage to the *SFL* on the 200 BC$_1$ plants originally included in the pools. Amplicons confirmed as being linked to *SFL*, together with one identified as being linked to *SFL* in a previous study (Cekic et al. 2001), were isolated and cloned as described below.

Isolation of the *SFL*-linked ISSR markers

ISSR primer combinations found to produce markers linked to *SFL* were applied to DNA of both parents (*F. vesca* f. *vesca* and *F. vesca* f. *semperflorens*). The resultant products were fractionated by low-temperature PAGE using 48S pre-cast polyacrylamide gels (Amersham, St. Albans, UK) as described by Charters et al. (1996), but omitting the final impregnation stage in the subsequent silver staining. *SFL*-linked fragments were gel excised and incubated in 30 μl TE buffer at 4°C for 16 h. An aliquot of 2 μl DNA from the eluted fragment was used as template for PCR, using the original ISSR primers and PCR conditions but with an additional 15 min final extension. The resultant re-amplification products were visualised on a 2% (w/v) agarose gel, and the product with lowest mobility in the profile was excised using the Qiaex II DNA extraction kit (Qiagen). Gel-extracted fragments were ligated into a pCR2.1-TOPO or pCR4-TOPO vector using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. There were ten white colonies selected for further analysis from each transformation. The plasmid DNA of selected *Escherichia coli* colonies was isolated using the QIAprep Spin miniprep kit (Qiagen). Colonies that gave rise to amplicons of the same size as the isolated band were subjected to cycle sequencing. At least three, and in some cases, ten such colonies from each transformation were sequenced using the ABI PRISM Dye terminator cycle sequencing reaction kit

Table 1 ISSR primer combinations used for the BSA. Primer combinations in *boldface* indicate the combinations that produced seasonal flowering locus (*SFL*)-linked candidate markers

Primer (5′→3′)		Primer combinations			
807	(AG)$_8$T[a]	1.	807+890	13.	835+891
808	(AG)$_8$C	2.	807+891	14.	888+807
810	(GA)$_8$T	3.	807+808	15.	888+808
811	(GA)$_8$C	4.	**807+810**	16.	888+810
835	(AG)$_8$YC	5.	**807+835**	17.	888+811
836	(AG)$_8$YG	6.	**807+836**	18.	888+835
841	(GA)$_8$YC	7.	**807+881**	19.	888+836
881	(GGGT)$_4$	8.	**881+889**	20.	888+841
888	BDB(AC)$_7$	9.	881+890	21.	888+842
889	DBD(AC)$_7$	10.	881+891	22.	888+881
890	VHV(GT)$_7$	11.	835+881	23.	**888+889**
891	HVH(TG)$_7$	12.	**835+889**		

[a] *Y* C or T; *B* C, G or T (not A); similarly *D* not C, *H* not G and *V* not T

Table 2 Oligonucleotide primers for *SFL*-linked markers ISSR1 and ISSR2 used to detect polymorphism in each locus

Marker	Primer name	Primer sequence (5′→3′)
ISSR1	P807+835 (F)	$(AG)_4$TCACATCCCG
	P807+835 (R)	$(AG)_5$TGAGGGGG
	835+A	$(AG)_8$TCA
	835+AC	$(AG)_8$TCAC
	835+ACA	$(AG)_8$TCACA
	807+G	$(AG)_8$TG
	807+GA	$(AG)_8$TGA
	807+GAG	$(AG)_8$TGAG
	N807+835 (F)	CACATCCCGGTTCTTAAGTC
	N807+835 (R)	GGGTGAAACTGATTTCTTACC
ISSR2	835+GG	$(AG)_8$CCGG
	889+AG	GGT$(AC)_7$AG

(PE Applied Biosystems) and fractionated on a 373 ABI automated sequencer (PE Applied Biosystems). Sequencing data were analysed using the Chromas software package (http://www.technelysium.com.au/chromas14x.html) for editing of sequence ambiguities and aligned using the ClustalW 1.8 software (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html).

Design of locus-specific primers

In the cases where the polymorphism between the parental alleles was caused by a base substitution (i.e. ISSR1 and ISSR3), locus-specific primers were designed to target the single-nucleotide polymorphism (SNP). Amplification was performed with primers designed so that their 3′ terminus was positioned on or near to the SNP and so resulted in a presence/absence detection of polymorphism. Use of appropriate control amplification was necessary to differentiate between failure of PCR and diagnostic absence of amplification caused by primer mismatch. In the remaining marker (ISSR2), polymorphism was attributed to amplicon size variation. Here, locus-specific primers were designed to accentuate the size difference between the parental alleles and so provide a co-dominant screen of the BC_1 population.

PCR conditions used for derivative primers (Table 2) were broadly similar to the original ISSR protocol. DNA amplifications were carried out in 20-μl reactions containing 20 ng template DNA, 0.5 U *Taq* polymerase (Roche), 2 μl reaction buffer (10× with 15 mM $MgCl_2$), 0.2 mM each dNTP and 0.3 μM each primer.

Linkage analysis with SCAR markers

Inheritance behaviour of SCAR markers was assessed on the 200 BC_1 progeny and compared with that of the original ISSR fragments. The linkage relationship of the newly derived SCAR markers and the *SFL* was determined using the JoinMap software version 2.0 (Stam 1993), using a LOD score of 3.0 and the Kosambi mapping function.

Results

Identification of the *SFL*-linked markers

The 23 selected ISSR primer combinations generated 345 amplicons (a mean of 15 per combination) when used for BSA on the seasonal-flowering and perpetual-flowering pooled samples. There were seven products that varied between pools and were therefore deemed to be candidate markers for the *SFL* locus (Table 1). When candidates were tested for co-segregation with *SFL* using the 200 BC_1 individuals included in the pools, only two markers from ISSR primer combinations 807+835 (marker ISSR1) and 835+889 (marker ISSR2) proved to be closely linked to *SFL* (Fig. 1). A third marker linked to *SFL* (ISSR3) identified in a previous study (Cekic et al. 2001) was derived from ISSR primer combination 835+841 (Fig. 1).

Cloning and sequencing of the *SFL*-linked ISSR markers

The three *SFL*-linked markers were cloned and sequenced. Each was gel excised from the ISSR profile of *Fragaria vesca* f. *vesca* after low-temperature PAGE and re-amplified using the same ISSR primers. Re-amplification of eluted fragments produced simplified band profiles comprising of 1–5 bands when visualised after electrophoresis through 1.5% (w/v) agarose gels. For ISSR3, the product with lowest mobility in the re-amplified profile was of similar inferred size to the corresponding progenitor band in the original ISSR profile (~320 bp). Re-

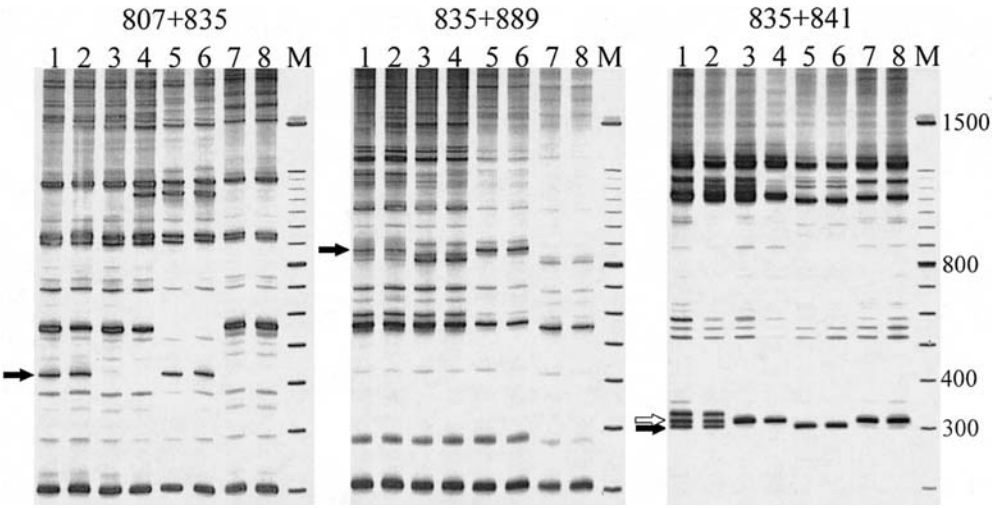

Fig. 1 ISSR banding profiles using 807+835, 835+889 and 835+841 primer combinations obtained on low-temperature PAGE gels. *Filled arrows* indicate *Fragaria vesca f. vesca* alleles in polymorphic loci, and an *open arrow* shows the *F. vesca* f. *semperflorens* allele generated by primer combination 835+841. *Lanes 1, 2* Seasonal-flowering BC_1 progenies; *lanes 3, 4* perpetual-flowering BC_1 progenies; *lanes 5, 6 F. vesca* f. *vesca* parent; *lanes 7, 8 F. vesca* f. *semperflorens* parent; *M* 100-bp ladder

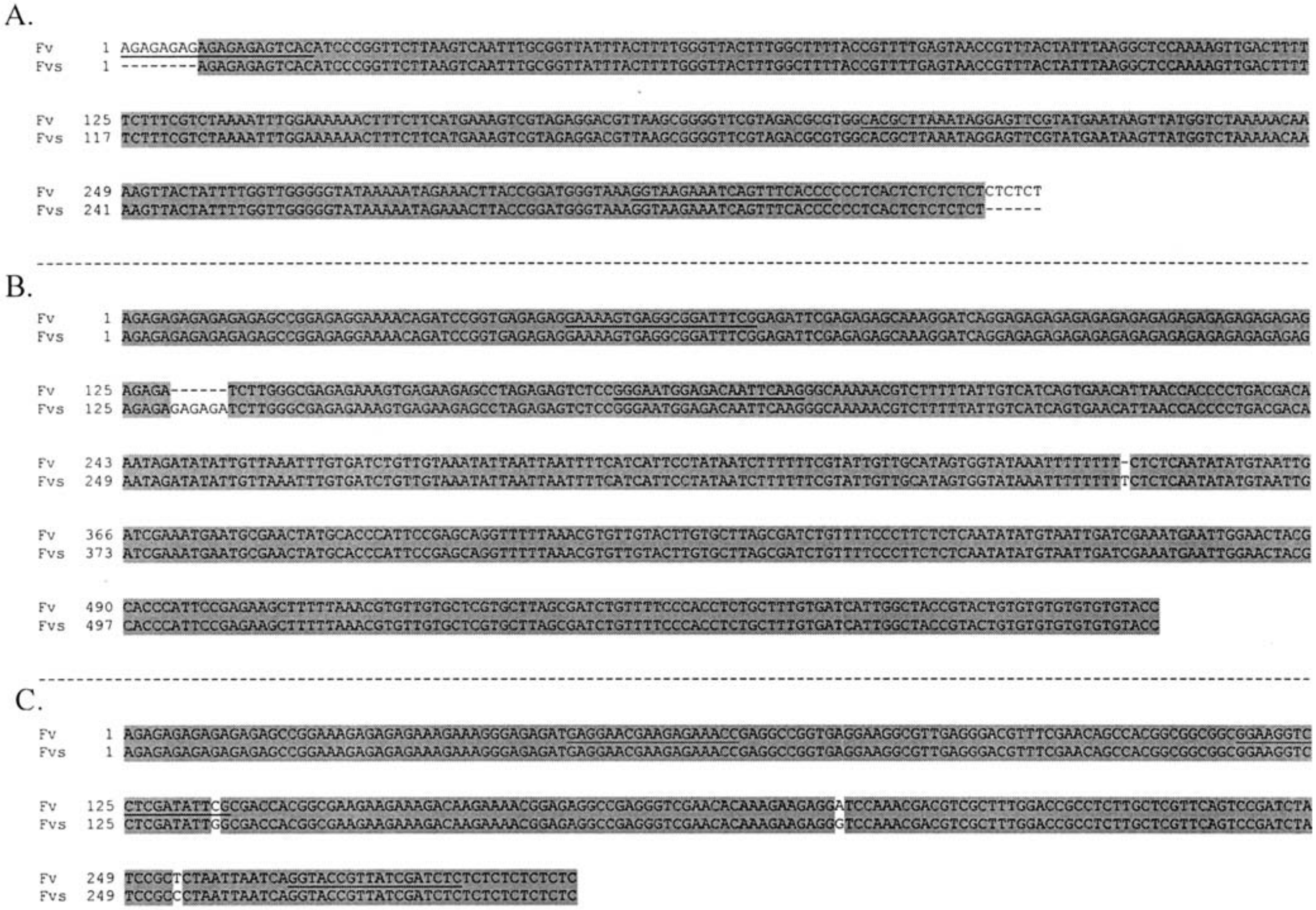

Fig. 2 Alignment of the parental alleles *F. vesca* f. *vesca* (*Fv*) and *F. vesca* f. *semperflorens* (*Fvs*) at **A**. ISSR1 **B**. ISSR2 and **C**. ISSR3 loci. *Underlined* sequences indicate positions of the forward and reverse sequence-specific primers used for the SCAR markers

amplification of the ISSR1 and ISSR2 markers generated amplicons of a lower inferred molecular weight after electrophoresis through agarose (~350 bp, ISSR1 and ~600 bp, ISSR2) than was estimated from the progenitor ISSR profile when fractionated by low-temperature PAGE (~430 bp and ~880 bp respectively). In all cases, the band with lowest mobility in each re-amplification profile was selected for cloning and subsequent sequencing using the M13 forward and reverse primers. Amplicon sizes inferred from the resultant trace data corresponded more closely with sizes predicted from agarose gels than estimates from ISSR profiles visualised after low-temperature PAGE (Fig. 2). As expected, primer-binding sites were discovered in the terminal sequences of all fragments.

Source of the original ISSR polymorphism

Polymorphisms in ISSR profiles are usually based on the presence/absence of a specific amplicon (band). In order to characterise the cause of differential amplification of specific products between genotypes, it is first necessary to generate homologous amplicons from the null genotype, in this case from *F. vesca* f. *semperflorens*. Locus-specific primers were therefore designed from sequence information of *F. vesca* f. *vesca.* These were used for PCR on both parents.

In the case of ISSR1, oligonucleotide primers [P807 +835 (F+R)] were designed such that the new primers included 10–11 bases from the 3′-end of the original ISSR primer and a seven- to eight-base 3′ extension, using the internal sequence from the amplicon (Table 2). The use of the P807+835 (F+R) primer combination generated a common amplicon. This product was cloned and sequenced from both parental genotypes. In total, 20 colonies (ten from each parent) were analysed, and all contained DNA inserts of the expected size (~340 bp). Cycle sequencing failed to reveal sequence differences between parents. This led to the inference that the polymorphism in the original ISSR profile was attributable to mutations at or around the binding sites targeted by the original ISSR primers. Single-nucleotide mutations of this kind may reside at either or both primer-binding sites. For this reason, the two original ISSR primer-binding regions were studied separately by exploiting intolerance of base mismatch at positions close to the 3′ terminus of the primer. Towards this end, we extended the original ISSR primers by one to three bases (primer 835+A, 835+AC, 835+ACA, primer 807+G, 807+GA and 807+GAG) based on amplicon internal sequence (Table 2). The ability of

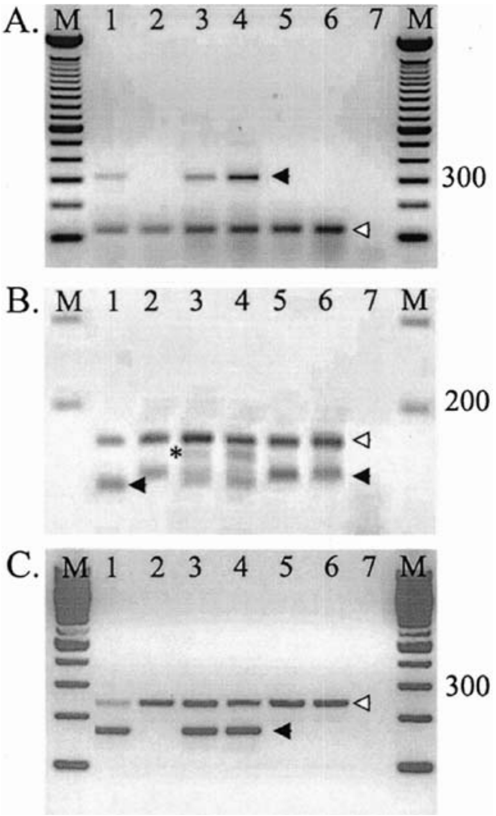

Fig. 3 Segregation of the SCAR markers (1–3) derived from the *SFL*-linked markers SCAR 1 (**A**), SCAR 2 (**B**) and SCAR 3 (**C**). In all cases, samples are loaded in the following order: *F. vesca* f. *vesca* parent (*lane 1*), *F. vesca* f. *semperflorens* parent (*lane 2*), seasonal-flowering BC_1 progenies (*lanes 3, 4*) and perpetual-flowering BC_1 progenies (*lanes 5, 6*). *Lane 7* is the negative control and *M* is the 100-bp ladder. In the dominantly inherited markers SCAR1 (**A**) and SCAR3 (**C**), polymorphic markers (*filled arrows*) are limited to samples containing a *F. vesca* allele. In the co-dominantly inherited SCAR2 marker (**B**), however, both parental alleles are visible (*filled arrows*). A heteroduplex band (*asterisk*) is seen in heterozygous samples. The *open arrows* indicate position of the control bands

these extended primers to yield a polymorphic PCR product was tested using locus-specific reverse primers [N807+835 (R) and N807+835 (F)] designed to target invariable internal regions of the locus (Table 2; Fig. 2A). Amplification using extended 807 primers (807+G, 807 +GA and 807+GAG) combined with the conserved return primer [N807+835(F)] failed to retain the original polymorphism. In contrast, amplification using extended primer 835 with a conserved reverse primer targeting the same internal site as above did generate polymorphic amplification products. For example, primer 835+AC produced a band that replicated the original ISSR polymorphism between the two parents (Fig. 3A). When the primers were extended further (835+ACA), polymorphism was also retained, although amplification was not as strong (data not shown). These results were expected if the original polymorphism was caused by a substitution in the 3′ anchor of the original ISSR primer 835.

A similar approach was used for the ISSR2 locus. The *F. vesca* f. *semperflorens* allele was sequenced and new primers created by extending the original ISSR primers by two bases (835+GG and 889+AG), using internal amplicon sequence information. These primers produced a single band in both parents of similar size (~600 bp) to ISSR2. This monomorphic fragment was subsequently cloned and sequenced. Sequence comparison revealed a clear and consistent difference between parents in the length of an interstitial 'GA' SSR (positions 92–135, Fig. 2B). The SSR of *F. vesca* f. *vesca* consistently contained three fewer repeats than that of *F. vesca* f. *semperflorens.* There was also a single base indel in a poly-T mononucleotide SSR that appeared between positions 339 and 347. This variation in size was insufficient to produce detectable differences when specific amplicons from the respective plasmids were fractionated on agarose, but when the same products were fractionated by low-temperature PAGE, the polymorphism observed in the original ISSR profile was retained (data not shown). Interestingly, even though sequence information revealed that the *F. vesca* f. *semperflorens* allele possessed an additional three GA repeats, it nevertheless exhibited a higher mobility than the *F. vesca* f. *vesca* allele when subjected to low-temperature PAGE. The presence of an intervening SSR between ISSR primer-binding sites was thereby tentatively identified as the probable cause of original ISSR polymorphism when separated by low-temperature PAGE. The apparent absence of the amplicon from the *F. vesca* f. *semperflorens* profile was attributed to its near co-migration with several other, unrelated amplicons. Indeed, there were four bands sufficiently closely associated with ISSR2 marker in the original ISSR profile when subjected to low-temperature PAGE to be considered as candidate homologues of ISSR2. The clustering of these candidates within the gel and their poor amplification rendered the direct cloning and sequencing of the ISSR2 homologue from *F. vesca* f. *semperflorens* profile impractical.

The ISSR3 marker in *F. vesca* f. *vesca* was closely associated with a band of similar intensity but very slightly different mobility in *F. vesca* f. *semperflorens*. The latter band appeared in profiles of all perpetual plants (*sfl*/*sfl*) in the BC_1 (the *F. vesca* f. *vesca* ISSR3 band was always absent) and so was tentatively identified as possibly representing the *F. vesca* f. *semperflorens* allele of ISSR3. This candidate was therefore gel excised from the *F. vesca* f. *semperflorens* profile, cloned and sequenced. The amplicons of *F. vesca* f. *vesca* (ISSR3) and *F. vesca* f. *semperflorens* showed 98% similarity, with differences noted in only three base substitutions at positions 134, 199 and 254. This was taken to indicate that the two products probably represent two parental alleles of the same locus (ISSR3).

Table 3 PCR-based markers linked to the *SFL* locus

Marker	Primer	Sequence (5′→3′)	PCR conditions
SCAR1	835+AC	AGAGAGAGAGAGAGAGTCAC	94°C, 1 min; 55°C, 2 min; 72°C, 30 s (35 cycles) and 72°C, 5 min
	N807+835 (R)	GGGTGAAACTGATTTCTTACC	
	C2 807+835 (F)	CACGCTTAAATAGGAGTTCG	
SCAR2	SSR2A	GAAAAGTGAGGCGGATTTCG	94°C, 1 min; 59°C, 2 min; 72°C, 30 s (35 cycles) and 72°C, 5 min
	Last2R	CTTGAATTGTCTCCATTCCC	
SCAR3	POLY4	GGAAGGTCCTCGATATTCG	94°C, 1 min; 63°C, 2 min; 72°C, 30 s (35 cycles) and 72°C, 5 min
	841CAP (R)	GAGATCGATAACGGTACC	
	COM1-SCAR3	GAGGAACGAAGAGAAACC	

Development of SCAR markers

ISSR1

Polymorphism at the ISSR1 locus seemed most likely to be caused by a point mutation in the region flanking the 835 primer-binding site. Extension of the 835 primer by two bases combined with a locus-specific primer N807+835 (R) resulted in a single amplicon that nevertheless retained the original polymorphism. ISSR1 marker was therefore converted to a SCAR1 marker using the extended original 835 ISSR primer (835+AC) combined with a locus-specific reverse primer N807+835 (R) to amplify a polymorphic band of 319 bp (Fig. 3A; Table 3). SCAR1 marker, a dominant marker, appeared as a single-band amplicon in the presence of the *F. vesca* f. *vesca* allele (*SFL/SFL* or *SFL/sfl*). Perpetual-flowering BC_1 progenies are homozygous for the *F. vesca* f. *semperflorens* allele (*sfl/sfl)* and so should not have given any amplification products when amplified with SCAR1-specific primers. A control was required, however, to ensure that the absence of amplification was not due to failure in the PCR reaction. A locus-specific internal primer C2 807+835 (F) combined with the N 807+835 (R) primer yielded an amplicon that was common to both parents (Table 3). Multiplex-PCR amplification using these three primers [835+AC, N807+835 (R) and C2 807+835 (F)] in a single mix resulted in two products: the polymorphic marker at 319 bp produced from the 835 +AC/N807+835 (R) primer combination, and a common amplicon 120 bp in length produced by the C2 807+835 (F)/N 807+835 (R) primer combination (Fig. 3A).

ISSR2

The ISSR2 amplicon contained an internal SSR that was polymorphic between the two parents and this was exploited to develop a locus-specific SCAR marker. Two locus-specific primers were designed to target regions flanking the variable SSR. The resulting amplicon was smaller than ISSR2 to accentuate the size difference between the two SSR alleles at the locus. Thus, SSR2A and Last2R produced a product of ~154 bp in which it was possible to distinguish the allelic states of the two parents after electrophoresis through agarose gel matrices, with the heterozygous backcross plants (seasonal flowering, *SFL/sfl*) exhibiting both alleles and also a heteroduplex band (Fig. 3B; Table 3).

ISSR3

Here, a new primer (POLY4) was designed such that the 3′ end targeted the C/G polymorphism between the parents at base position 134 (Fig. 2C; Table 3). Amplification using primer POLY4 combined with a conserved internal reverse primer 841CAP (R) yielded a distinct polymorphic fragment of about 170 bp in the presence of the *F. vesca* f. *vesca* allele and so apparently retained polymorphism between the two parents (Fig. 3C; Table 3). SCAR3 marker was therefore based on the differential ability of the two parents to generate amplification products, and so a control amplicon linked to the *SFL* locus was used to differentiate nulls from amplification failure. A conserved primer COM1-SCAR3 was therefore designed based on the internal sequence of the SCAR3 amplicon (Table 3). When the COM1-SCAR3 primer was combined in a single mix with POLY4 and 841CAP (R) primers, a common 240 bp fragment was produced along with the polymorphic 170 bp fragment.

Local mapping of the *SFL* using the SCAR markers

The three locus-specific markers (SCAR1, 2 and 3) developed in this study were mapped in relation to the *SFL* gene. The SCAR markers were assessed in a blind screen in the 200 individual BC_1 progenies that had been previously used in the pools and so had been genotyped using the corresponding progenitor ISSR markers. Simple amplicons were able to identify recombinant individuals in a dominant manner (i.e. presence/absence of a polymorphic band) for SCAR1 and SCAR3 and in a co-dominant fashion (i.e. small-length polymorphism between seasonal-flowering and perpetual-flowering individuals) after fractionation by electrophoresis in 2% (w/v) agarose gels for SCAR2. All recombinants identified using the SCAR markers were identical to those identified in the original ISSR profiles, confirming that the locus-specific markers had targeted the correct loci and had also retained the original polymorphism. A local map was therefore

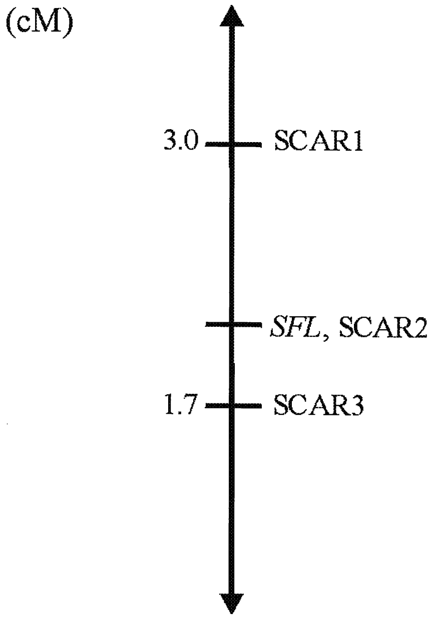

Fig. 4 Local map around the *SFL* in *F. vesca* based on 1,049 BC_1 progenies

constructed using these SCAR markers based on 1,049 BC_1 progenies (Fig. 4). SCAR2 was mapped to the same position as the *SFL* gene, whereas SCAR1 was located on the north side of the gene at the genetic distance of 3 cM, and SCAR3 was positioned 1.7 cM to the south of the gene.

Discussion

The *SFL* gene is inferred from physiological observations to be a floral repressor that is inactivated by short days and cool temperatures in the autumn but reactivated by winter cold (Battey et al. 1998). The gene thereby controls the seasonal reversion from flowering to vegetative growth in *Fragaria vesca*. This ability is restricted to polycarpic perennials, and so its control is difficult to predict from candidate genes isolated from model annual species. For instance, *FLOWERING LOCUS C*, a floral repressor from *Arabidopsis thaliana* (Michaels and Amasino 1999; Sheldon et al. 1999) is one possible candidate for *SFL*, although flowering responses of this gene to low temperatures in *Arabidopsis* appear to be opposite to that seen in *Fragaria* (Battey 2000). Floral promoters from the *A. thaliana* flowering-time network may also be candidates for a repressor function in *Fragaria.* Whilst candidate gene studies report the presence of several genes that regulate flowering in *A. thaliana,* in some perennial species [e.g. apple, Wada et al. (2002); poplar, Rottmann et al. (2000); *Lolium perenne*, Jensen et al. (2001); *Pharbitis nil*, Kim et al. (2003)], the function of these homologues has not been clearly demonstrated.

Positional cloning of *SFL* is therefore desirable to facilitate a better understanding of the regulation of flowering in the perennial life cycle of *F. vesca*. Here, we developed locus-specific markers from three ISSR amplicons linked to *SFL* to assist large-scale screening of the mapping population for future positional-cloning efforts.

The process of cloning and sequencing the *SFL*-linked fragments from ISSR profiles proved to be problematic. We found that re-amplification of the ISSR fragments excised from pre-cast polyacrylamide gels after low-temperature PAGE usually generates one or more products of lower mobility in addition to the target fragment. The extent of this contamination seems to depend partly on the intensity and position of the excised fragment in the original PAGE profile. For example, the ISSR2 marker appeared as a minor band of relatively low mobility in the original PAGE profile and produced more contaminant lower-molecular-weight bands upon re-amplification than did the strongly amplified, high-mobility fragments ISSR1 and ISSR3. Low-temperature electrophoresis as applied here can have a profound influence on DNA migration rates (Orita et al. 1989) such that it can be difficult to relate the position of a target in the original profile to that occupied after agarose gel electrophoresis. This may explain why re-amplified ISSR1 and ISSR2 markers appeared to be of lower-molecular weight when estimated on agarose gels than originally inferred following low-temperature PAGE. Hauser et al. (1998) reported that high-resolution matrices (in their case, the commercially available MDE-mutation detection enhancement gel) are sensitive to differences in the base composition of the DNA fragments and cause differences in their electrophoretic mobility, with a discrepancy of almost 100 bp being noted in the mobility of some fragments of known size. For some targets, particularly those of low mobility in complex profiles, such differences between the properties of high resolution and agarose fractionation may exacerbate difficulties in identifying and isolating the target prior to cloning.

In the present study, the three targeted loci exhibited contrasting causes of polymorphism. The marker designated ISSR1 appeared invariant between parents for internal sequence, and polymorphism was only retained when one of the ISSR primers was lengthened slightly and used in combination with a common internal reverse primer. Accordingly, the cause of the original polymorphism was attributed to sequence differences between the parents in the 835 primer-binding site. However, it is not possible to distinguish with certainty whether this mutation occurred in the anchor or SSR components of the ISSR primer. Variation in the internal sequence was observed for the remaining markers (ISSR2 and ISSR3). In the case of ISSR2, the presence of an SSR that differed in size between parental lines almost certainly caused the original ISSR polymorphism. Other workers have reported similarly polymorphic SSRs residing within internal sequences of AFLP (e.g. Meksem et al. 2001; Wong et al. 2001) and RAPD amplicons (Bautista et al. 2003). The detection of size variation attributable to internal SSRs depends on the resolution of the fractionation system, the scale of the length polymorphism and amplicon length. In the current work, the use of high resolution pre-cast gels for low-temperature PAGE provided sufficient resolution for the clear separation of both alleles in the original ISSR profile, whereas agarose-gel electrophoresis was sufficient to discriminate alleles of the derivative SCAR marker. For the final marker, ISSR3, products were amplified from both parents but each showed a slightly different mobility.

This suggested that the original ISSR polymorphism was caused by amplicon size variation or by internal sequence differences. When the parental amplicons were cloned, both were of identical length and differed by just three point mutations. These minor differences must therefore have differentially influenced fragment migration during low-temperature PAGE. The use of urea in the loading buffer (Charters et al. 1996) means that the ISSR amplicons were probably dissociated into the single-stranded form prior to electrophoresis. Fragment dissociation may be implicated in the original ISSR polymorphism observed for this marker. Minor changes to the sequence of a single-stranded DNA fragment can influence the manner in which it folds upon itself, particularly at low temperatures, and this in turn affects mobility through a gel matrix. This principle has been widely exploited in single strand conformational polymorphism analysis where small differences in sequence between the alleles of target genes are used to generate amplicons with disparate mobility when fractionated at low temperature (Hunger et al. 2003).

The probability of failure when attempting conversion of a variable ISSR band into a locus-specific SCAR marker is reduced if the cause of the original polymorphism is first characterized. Once this objective has been achieved, the most appropriate strategy can be elected for conversion. For instance, length variation between alternate alleles of SCAR2 was simply exposed by the design of locus-specific primers flanking the internal SSR. In this situation, one pair of primers generates both alleles and so the resultant SCAR marker is co-dominantly inherited. A different approach was required where the original band polymorphism appeared to be based on variation in internal sequence (SNP) rather than amplicon length (e.g. SCAR3). There are many strategies for exploiting SNP variation, but here we used a simple, low-cost approach based on the design of primers such that the SNP lies at the 3′ terminus. In this way, the strength of amplification using primers that target the alternative alleles can be compared to infer genotype. The use of differentially labelled primers allows competitive PCR to determine genotype in a single reaction. Kanazin and coworkers (2002) used this strategy to detect informative SNPs that differentiate several barley varieties. The discovery of polymorphisms caused by point mutations at or close to the 3′ end of the original ISSR primer presents more of a problem for conversion to SCAR. On the one hand, extension of both primers is likely to generate locus-specific amplification, but on the other, primer extension also risks losing the original polymorphism as the position of the variable base is forced away from the 3′ end of the new primer. Indeed, in the current study, significant extension of both ISSR primers at the ISSR1 locus generated common amplicons in which both alleles possessed identical internal sequence. We therefore elected to slightly extend each of the ISSR primers in turn. This approach enabled retention of the original polymorphism whilst also generating a single amplification product.

Overall in this study, we have developed three SCAR markers linked to the *SFL* gene that will have utility in the positional cloning of the *SFL* gene and for transfer of the trait into cultivated *F. vesca* clones by marker-assisted selection. The markers may also have value in *F. vesca* for map-based screening of the many potential candidate homologues described from model species such as *A. thaliana*.

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