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PCR-based Chromosome Walking: The *SFR 3* Case

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Abstract: In this study, mapping experiments were initiated in a region of 34.23 cM or 2.7 Mb to map the *SFR3* gene into the smallest possible region on chromosome I. Eleven new PCR markers had been designed to fine map the gene. Roughly 557 F2 plants tested in these experiments and 38 lines were then used in the fine mapping experiments. The fine mapping experiments left the *SFR3* region amongst 26 genes. Finally, 13 pseudogenes and transposable elements were eliminated and this left the *SFR3* gene region amongst 13 genes in a 0.19 Mb region, which is represented by 4 BAC clones. The PCR-based chromosome mapping experiments to fine map the *SFR3* gene have successfully decreased the *SFR3* region from 2.7 to 0.19 Mb. We concluded that PCR-based chromosome walking is a convenient method to clone *SFR3* gene.

Key words: PCR-based chromosome walking, *SFR3* mutant, freezing sensitive

INTRODUCTION

In the last two decades a number of *Arabidopsis* mutants have been identified which show an altered response to cold acclimation and have been used in studies of stress tolerance in *Arabidopsis*[1-3].

The *SFR3* mutant is known to be sensitive to freezing temperatures and does not accumulate anthocyanin at 4°C during cold acclimation. The *SFR3* mutation causes the greatest freezing-sensitivity in young (incompletely expanded) leaves. It is indistinguishable from wild type at normal growth temperatures, but displays two pleiotropic effects at 4°C: In the short term (during normal cold acclimation) mutant plants are deficient in anthocyanin and in the long term (approximately 8 weeks and above) accelerated senescence of older leaves[4].

The *SFR3* mutation has no effect on cold-induced gene expression[5]. This makes the *SFR3* mutation interesting as it may help to elucidate a specific individual mechanism for preventing freezing injury distinct from the DRE/CRT pathway[6].

Initial mapping of *SFR3* by Thorlby *et al*.[7] placed it on chromosome I, between the markers UFO and GAPB.

Chromosome walking, or map based cloning, is one of the techniques to clone genes from loss-of-function mutants.

Positional cloning has been acknowledged as a tedious technique[8] as it requires a large amount of good quality DNA, as well as considerable time from sample analysis to results (in case of RFLP [restriction fragments length polymorphisms]). However, with PCR techniques many new PCR markers have been developed, which are more convenient, require less DNA and so lead to a more rapid analysis. In these cases crude DNA can be used, PCR performed and the products run on gels to detect polymorphisms. The common PCR markers used are SSLP (simple sequence length polymorphisms)[9] and CAPS (cleaved amplified polymorphic sequences) markers[10]. The SSLP marker amplifies regions of microsatellite DNA that differ in length, while CAPS markers amplify regions of DNA containing a restriction enzyme site unique to an allele.

Previous studies localised the *SFR3* gene on chromosome I within a region comprising 10 BACs (F21H2 to F5J5) (Dr. G. Warren, personal communication). In this study, mapping experiments were initiated in a region of 34.23 cM or 2.7 Mb to confirm the previously found map location (Fig. 1).

MATERIALS AND METHODS

Plant material and growth conditions: Plants from ecotypes Columbia and Landsberg erecta of *Arabidopsis thaliana* L. (Heynh) were used in this study. However, the background for *SFR3* (sensitive to freezing) mutants was the ecotype Columbia. Plants were grown under a long day period to get early flowering. They were grown

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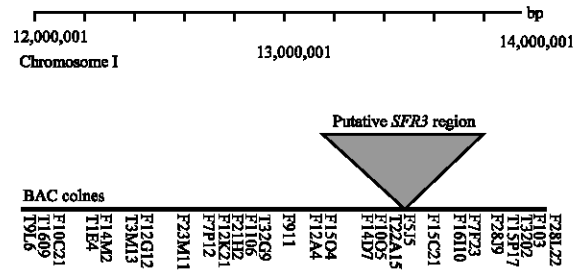

Fig. 1: The putative *SFR3* region on chromosome I

on compost (Levington) under a light intensity of 250 μmol m^{-2} s^{-1} at 22°C from cool-white fluorescence lights with a 16 h light and 8h dark cycle. Humidity in the room was maintained at 60 to 70%.

To generate recombinants, *SFR3*$^{-}$/*SFR3*$^{-}$ (Columbia) plants were crossed with *SFR3*$^{+}$/*SFR3*$^{+}$ (Landsberg). F2 plants from this cross were used for mapping experiments.

Seeds were collected when siliquas had becomes dry and brown (approximately 2 weeks from first day after flowering). They were shaken onto paper. Seeds were passed through a sieve (0.5 mm) to remove debris and stored at room temperature in glass tubes with holes in lids to prevent excess humidity.

For freezing tests, short day conditions were applied; plants were grown on compost under a light intensity of 170 μmol m^{-2} s^{-1} at 22°C from cool-white fluorescence lights with a 10 h light and 14 h dark cycle. Forty-eight plants were grown together with the *SFR3* mutant and wild type Columbia in the same under short day conditions. They were then transferred into the cold room for 10-12 days for cold acclimation and finally transferred into a freezer at -5.2 to -6.2°C overnight. The plants were then moved to the dark for 4 h at 22°C prior to short day conditions. Results were observed by visual observation of the injured young leaves 3-4 days after the final short day treatments.

Preparation of plant genomic DNA for PCR: DNA suitable for PCR was isolated from flower buds and leaves using the method of Edwards *et al.*[11]. The unopened buds from F2 plants that planted under long day condition were snipped off carefully from the main stem and from any side shoots to ensure buds were not accidentally taken from neighboring lines and put into a microcentrifuge tube. The tissues were ground for approximately 10 sec using a micro pestle. Then, 400 μL Edwards buffer (200 mM Tris-Cl, pH 7.5, 250 mM NaCl, 250 mM EDTA, pH 8.0, 0.5 % w/v SDS) was added to the sample and vortexed for 5 sec. Sample was then centrifuged at maximum speed (13,000 rpm) for 1 min in a microcentrifuge (5415 D, Eppendorf). Supernatant (300 μL) was transferred into a fresh microcentrifuge tube and 300 μL isopropanol added. The mixture was left at room temperature for 2 min. Finally, the DNA was collected by centrifugation (5 min at 13,000 rpm) and the pellet was air dried before resuspension in 100 μL dH_2O or TE (1 M Tris pH, 8.0 and 0.5 M EDTA) and stored in a fridge (0 to 4°C).

Primer design and synthesis: Primers for PCR and sequencing were designed using Primer3.cgiv0.2c software (http://www.genome.wi.mit.edu). The default setting was used except for conditions where no primer was successfully designed so a few changes (e.g., primer size and temperature) were made according to the statistical reports prepared by the software programme. Primers were synthesised by MWG Biotech, Germany.

Finding the crossover lines between F21H2 and T27K12: Isolation of DNA from 557 F2 plants was carried out as described above. DNA samples were then tested by PCR, as described above and the PCR products analysed by either agarose gel or PAGE. Each of the F2 lines was tested with two flanking PCR markers, F21H2 and T27K12 (2.7 Mb), to find recombinants. The *SFR3* region was believed to be located at the right of F21H2 and left of T27K12 (Dr. G. Warren, personal communication).

Designing molecular markers, fine mapping of the *SFR3* region and freezing tests: A number of PCR markers (SSLP or CAPS) were designed to carry out mapping experiments using various public databases. The Cereon homepage[12] was used to find information regarding polymorphism between *Arabidopsis thaliana* ecotype Columbia and Landsberg. Sequences containing these polymorphisms were obtained from the MIPS databases[13] and used to design PCR primers. Primers were synthesised by MWG Biotech, Germany. Beside the new markers, six known markers including F21H2 and T27K12, were used in these experiments. The newly designed primers were tested using control DNA (DNA isolated from Columbia and Landsberg plants) for optimisation of PCR conditions and their reliability to detect polymorphisms. A few changes of annealing temperature, extension time and magnesium concentration were carried out for these optimization according to the primer.

New markers, which produced amplicons that proved to have polymorphisms, were used for these mapping experiments. For CAPS markers, PCR products were digested with appropriate enzymes (Table 1) prior to electrophoresis. The information regarding restriction enzymes that could cut the amplicons at the

Table 1: List of PCR-based designed in the study and known PCR-based, with details of PCR conditions and the polymorphism

PCR marker	Printer sequence	MgCl (nM)	dNTPs (mM)	ATT	ET	Gel type	Polymorphism	Other special conditions
UFO	L-5'-GTGGCGGTTCAGACGGAGAGG-3'	2.5	0.2	56°C	2 min	6%	Col: 983, 316	Cut with *Taq*1
	R-5'-AAGGCATCATGACTGTGGTTTTTC-3'			30 sec		page	Ler: 600, 383, 316	at 65°C
F21H2	L-5'-GGGTCATCTTTTCTCTCTCC-3'	2.5	0.4	56°C	3 min	1%	Col: 1030	
	R-5'-TTCCATCGTTAGTCACAAGC-3'			30 sec		agarose	Ler: 555	
T9L1	L-5'-CAAAGAATGTCAAATACAAAATATCAC-3'	2.5	0.2	56°C	30 sec	6%	Col: 177	Run for a longer
(AZ1)	R-5'-TTCCTCAGGTTTGGCTCTTC-3'			30 sec		page	Ler: 121	time
Mi342	L-5'- GAAGTACAGCGGCTCAAAAAGAAG-3'	2.5	0.2	54°C	2 min	6%	Col: 400, 350	Cut with *Hinf*1
	R-5'-TTGCTGCCATGTAATACCTAAGTG-3'			30 sec		page	Ler: 340, 350, 50	at 37°C
SGCSNP	L-5'-TGCACCATAACCCTAAACCTG-3'	2.5	0.2	56°C	2 min	6%	Col: 350, 176	Cut with Dra1 and
161 (AZ5)	R-5'-GTGTCTTCCCAAACCGAATG-3'			30 sec		page	Ler: 526	run for a longer time
F10O5	L-5'-CTCTCCATCCCTGTCTGTACG-3'	2.5	0.2	56°C	1 min	6%	Col: 252	Run for a longer time
(AZ7)	R-5'-TTTCACGAAAACATCTCATTTTG-3'			30 sec		page	Ler: 218	
T22A15	L-5'-TTTCCCGGGCTTTAATTAGC-3'	2.5	0.2	56°C	1 min	6%	Col: 354	Run for a longer time
(AZ9)	R-5'-CAAAACTCCAAAAACTAAATCATAAAC-3'			30 sec		page	Ler: 396	
F5J5	L- 5'-TCCAACCATATCTTCACGTAACC-3'	2.5	0.2	54°C	3 min	6%	Col: 373, 292	Cut with *BstE*11
(AZ11)	R-5'-TGCCCAATACCAGGACTATG-3'			25 sec		page	Ler: 337, 217	at 60°C
F5J5-2	L-5'-AAGGATCCGCGTGTTTTTAG-3'	2.5	0.2	54°C	3 min	6%	Col: 598, 90, 71 ,33	Cut with *Dra*1
(AZ12)	R-5'-TGATCCGACGGTTAAGAAGG-3'			30 sec		page	Ler: 598, 104, 90	
F7F23	L-5'-CAGAAAATAAACGTGTGTATCAAAG-3'	2.5	0.2	56°C	1 min	6%	Col: 339	Run for a longer time
(AZ10)	R-5'-GCACATTATTTATGCGGGTTG-3'			30 sec		page	Ler: 308	
F28J9R	L-5'-CACTCCCTGATAACCCAACC-3'	2.5	0.2	56°C	3 min	6%	Product 350 bp	Only useful to differentiate
AZ8)	R-5'-GGTGTTTTTCGAAATTCTCCTTC-3'			30 sec		page	Het has magic band.	between Het. and Ler
T15P17	L-5'-CCCAATCCCGTAACTGAAAG-3'	2.5	0.2	56°C	1 min	6%	Col: 260	
(AZ6)	R-5'-GGCATTTCCAAAGAAGTAGACAC-3'			30 sec		page	Ler: 298	
T32E20	L-5'-TGGATTAGATGAAAGATGTAAAAGAAG-3'	2.5	0.2	56°C	1 min	6%	Col: 260	Run for a longer time
(AZ4)	R-5'-GCGACAGTCAAACGAACAAC-3'			30 sec		page	Ler: 228	
F28L22	L-5'-AAAATTAGTAATACAAAAATCGTGGTG-3'	2.5	0.2	52°C	1 min	6%	Col: 250	Run for a longer time
ta (AZ2)	R-5'-AAACTAGAACCAGGGGTAAAAGG-3'			25 sec		page	Ler: 201	
T27K12	L-5'-GGACAACGTCTCAAACGGTT-3'	2.5	0.2	56°C	2 min	6%	Col: 175	Run for a longer time
	R-5'-GGAGGCTATACGAATCTTGAC-3'			30 sec		page	Ler: 200	
Jcc3	L-5'-GGCTACTGGTCAAAATCATTC-3'	2.5	0.2	56°C	2 min	6%	Col: 225, 205	Cut with *Hsp9211*
	R-5'-GAATCTTTGCAAACGAGTGG-3'			25 sec		page	Ler: 250, 225	at 37°C
nga280	L-5'-CTGATCTCACGGACAATAGTGC-3'	2.5	0.2	56°C	2 min	6%	Col: 105	
	R-5'-GGCTCCATAAAAAGTGCACC-3'			30 sec		page	Ler: 85	

polymorphisms sites was obtained from the Cereon database. The new markers were designed to decrease the size of the BACs region by moving it inwards from the region of 54 BACs (from F21H2 to T27K12 markers) to 4 BAC.

The fine mapping experiments were subsequently complemented by freezing tests. In these experiments, seeds were collected from plants with a crossover in the region between the two markers (F21H2 and T27K12). Each line, were then cold acclimated and freeze tested. The tests were carried out once for each line. The genotype of F2 crossover plants was determined in these experiments.

The map distances of PCR markers were calculated by applying the Kosambi equation[14]. In this equation, r is the observed recombination, M_k is the estimated map distance and to obtain the value in centimorgan (cM), M_k is multiplied by 100. Observed recombinations were calculated by dividing the number of recombinants with the number of gametes involved in the experiments.

$$M_k = \tfrac{1}{4}[\ln(1 + 2r) - \ln(1-2r)]$$

$$cM = M_k \times 100$$

RESULTS

F21H2 and T27K12 crossover lines: Out of 557 F2 plants tested only 38 lines proved to have crossovers between the two flanking markers (F21H2 and T27K12). These 38 lines were then used in the fine mapping experiments.

***SFR 3* fine mapping:** Out of 26 new PCR markers (either SSLP or CAPS) 11 of them gave clear polymorphisms between DNA from *SFR3*/*SFR3*$^-$, heterozygotes and Landsberg (Table 1). Details of PCR conditions and polymorphisms that successfully showed polymorphisms are recorded in Table 1. Figure 2 shows example of gel showing bands from Columbia, Landsberg and heterozygous PCR products with PCR markers mi342.

Seeds from these 38 lines were grown and freeze tested. The *SFR3* gene might locate between PCR markers AZ11 and AZ10 (Table 2). The UFO and nga280 markers were used just to confirm that the map orientation was in the right *SFR3* region. The numbers of recombinants observed from the DNA tests with the 17 PCR markers were then used to calculate map distance. Table 3 shows a summary of the map distance calculations. These map distances were used in Fig. 3 and 4 that show a summary

Table 2: PCR results from 38 lines DNA tested with 17 PCR markers and freezing tests results

	PCR marker																
Line	UFO	F21H2	AZ1	Mi342	AZ5	AZ7	AZ9	AZ11	AZ12	AZ10	AZ8	AZ6	AZ4	AZ2	T27K12	Jcc3	nga280
311	C	C	C	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**
320	H	H	H	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	H
337	C	C	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**
350	H	H	H	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**
366	H	H	H	H	H	H	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**
387	C	C	C	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**C**
476	C	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	L	L	L	L	L	L	L
477	L	L	L	L	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	C	C
483	L	L	L	L	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**
484	L	L	L	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**
485		**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	L	L	L
494		L	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**
498	H	H	H	H	H	H	H	H	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**
503	C	C	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**
505		H	H	H	H	H	H	H	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**
519	L	H	H	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**
520	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	H	H	H
522		L	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**
530	L	L	L	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**
541	C	C	C	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	L
548	H	H	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	H
606	H	C	C	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	C
613	L	L	L	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**
620	L	L	L	L	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	
621	C	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	L	L	L	L	L	L
634	H	H	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	H
661	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	L	L	H
665	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	L	L	L	L	L	L	L	H
720	C	C	C	C	C	C	C	C	**H**	**H**	**H**	**H**	**H**		**H**	**H**	**H**
745	H	H	H	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**
762	C	C	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**
775	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	L	L
786	H	H	H	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**
799	H	H	H	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**
806	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	H	H
807	C	C	C	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**
812	C	C	C	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**
832	C	C	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**

C stands for Columbia, L for Landsberg and H for heterozygous. Bold regions indicate where the *SFR3* might locate. Final results have shown that *SFR3* gene locates between PCR markers AZ11 and AZ10

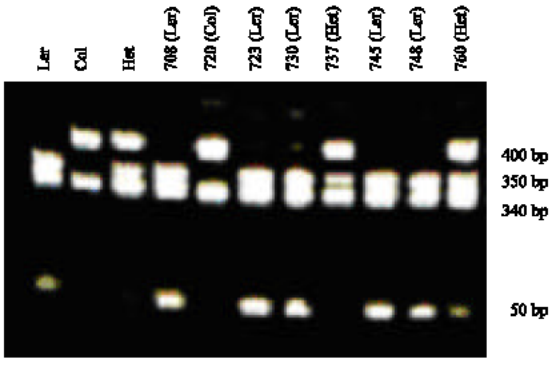

Fig. 2: 6% PAGE gel of PCR products from mi342 PCR marker. Example of some lines tested with the mi342 PCR marker. Ler stands for Landsberg, Col for Columbia and Het for heterozygous. Landsberg band sizes are 350, 340 and 50 bp, while Columbia band sizes are 400 and 340 bp

Table 3: A summary of observed recombinations between marker and *SFR3* and map distances

PCR Marker	A	Gametes (A)	B	C	D
F21H2	557	1114	30	0.027	2.7
AZ1	557	1114	22	0.019	2.0
Mi342	557	1114	7	0.006	0.6
AZ5	557	1114	4	0.003	0.4
AZ7	557	1114	4	0.003	0.4
AZ9	557	1114	3	0.002	0.3
AZ11	557	1114	3	0.002	0.3
AZ12	557	1114	0	0.000	0.0
AZ10	557	1114	1	0.001	0.1
AZ8	557	1114	2	0.002	0.2
AZ6	557	1114	3	0.002	0.3
AZ4	557	1114	3	0.002	0.3
AZ2	557	1114	3	0.002	0.3
T27K12	557	1114	8	0.007	0.7

A: Individuals tested with stated PCR marker; B: No. of recombinants observed from the test (B); C: Observed recombination = B/A
D: Best estimate of separation from *SFR3* (cM)

of fine mapping with all new PCR markers and their distances in cM and Mb.

Fig. 3: Mapping of the *SFR3* gene to a region of chromosome I from 38 BACs clones.

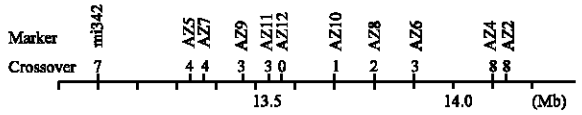

Fig. 4: Fine mapping of the *SFR3* gene to a region of chromosome I from 14 BACs clones.

The fine mapping experiments finally left the *SFR3* region amongst 26 genes. From database searches, 13 pseudogenes and transposable elements were eliminated and this left the *SFR3* gene region amongst 13 genes in a 0.19 Mb region, which is represented by 4 BAC clones (Table 4).

Table 4: The 13 genes in the *SFR3* region after elimination of the possible pseudogenes and transposable elements

BAC name	MIPS code number	Description
F5J5	At1g36150	Hypothetical protein
F15c21	At1g36160/70	Acetyl-CoA carboxylase
	At1g36180	Acetyl-CoA carboxylase
	At1g36230	Hypothetical protein
	At1g36240	60s ribosomal protein L30, putative
	At1g36280	Adenylosuccinate lyase-like protein
F7F23	At1g36310	Hypothetical protein
	At1g36320	Hypothetical protein
	At1g36340	Putative ubiquitin conjugating enzyme
	At1g36370	Putative hydromethyltransferase
	At1g36380	Hypothetical protein
	At1g36390	Putative heat shock protein
	At1636410	Hypothetical protein

DISCUSSION

The decision to use the known PCR markers (UFO and nga280) that lie far outside the previous *SFR3* region was useful because it demonstrated that the previous mapping of the *SFR3* region was accurate. As mapping of *SFR3* gene moved inwards from these markers, results suggested that the gene was actually to the right of the F5J5 not to the left of F5J5 (Fig. 1) in 4 BAC regions between F5J5 and F7F23 BAC clones (Table 2) or between AZ11 and AZ10 PCR markers (Fig. 4). This does not agree with previous mapping results, which suggested that *SFR3* region is between F21H2 and F5J5 BAC clones (Dr. G. Warren, personal communication). The reason for these differences is unknown, may be the F2 lines that they tested were insufficient to locate the *SFR3* regions.

In the case of the CAPS marker, failure to obtain any polymorphisms may-be because of a database error in the DNA sequence. In most cases the restriction enzymes suggested in the databases [e.g. PCR markers SGCSNP279 and CER431851 (data not included)] did not digest these fragments. In addition, some of the markers designed required extensive optimization of the PCR conditions. For example, with the SSLP primer the fragments amplified were too small (e.g. PCR marker AZ10) to show clear differences in size. For example, if the database suggested that the differences in size for Lansdberg and Columbia DNA sequence is 25 bp, the maximum size of PCR product must be not more than 200 bp to give clear polymorphisms using 6% PAGE. Unfortunately, PCR always gave more than one band when small size amplicons were amplified, so more time and effort was taken to optimize the extension time and sometimes the $MgCl_2$ concentration for this primer.

Experience shows that designing PCR markers is the crucial part in PCR-based mapping experiments. The size of amplicon for the SSLP marker should correspond to the length of the polymorphisms in microsatellite loci. If the length of polymorphisms is short, then the size of the amplicons should also be small, thus making detectable differences in amplicon size easier.

In contrast, amplicons from CAPS markers should be larger in size such that digestion products can still be seen clearly as two distinct bands. However, location of the polymorphisms must not be in the middle of the amplicon because this will give two bands with the same size.

Chromosome mapping experiments to fine map the *SFR3* gene have successfully decreased the *SFR3* region from 2.7 to 0.19 Mb. This implies that map based cloning using PCR markers accompanied with the *Arabidopsis* public database has changed map based cloning from tedious and laborious techniques to more convenient one, allowing analysis to be done in a shorter time. For future work, it would be preferable to start by planting many F2 lines (more than 1000 lines in our case) in order to get more recombinants. With a big number of recombinants, the target gene region can be mapped as finely as possible (100 kb). Faster methods for preparing DNA samples (not by using plastic pestle for grinding) would be very useful to allow many F2 lines to be tested in a short time.

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