Chapter 2

Quantification and Sequencing of Crossover Recombinant Molecules from *Arabidopsis* Pollen DNA

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Abstract

During meiosis, homologous chromosomes undergo recombination, which can result in formation of reciprocal crossover molecules. Crossover frequency is highly variable across the genome, typically occurring in narrow hotspots, which has a significant effect on patterns of genetic diversity. Here we describe methods to measure crossover frequency in plants at the hotspot scale (bp–kb), using allele-specific PCR amplification from genomic DNA extracted from the pollen of F_1 heterozygous plants. We describe (1) titration methods that allow amplification, quantification and sequencing of single crossover molecules, (2) quantitative PCR methods to more rapidly measure crossover frequency, and (3) application of high-throughput sequencing for study of crossover distributions within hotspots. We provide detailed descriptions of key steps including pollen DNA extraction, prior identification of hotspot locations, allele-specific oligonucleotide design, and sequence analysis approaches. Together, these methods allow the rate and recombination topology of plant hotspots to be robustly measured and compared between varied genetic backgrounds and environmental conditions.

Key words Meiosis, Recombination, Crossover, Allele-specific PCR, Titration, Pollen DNA

1 Introduction

Meiotic recombination is conserved throughout the majority of eukaryotes, and has importance for human fertility, agricultural breeding, and understanding patterns of genetic diversity [1–4]. During meiosis, programmed DNA double strand breaks are induced by SPO11, coincident with pairing of homologous chromosomes [3, 5, 6]. The broken DNA ends are then repaired using the homologous chromosome as a template, which can result in reciprocal exchange, or "crossover" [3, 5–7]. Crossover numbers and distributions are under tight genetic control, usually consisting of one or a small number occurring per chromosome arm, regardless of the physical size of the chromosome [7]. Meiotic recombination rate is also highly heterogeneous along chromosomes, with many species having narrow "hotspots", typically kilobases (kb) in width, with high recombination rates relative to the genome

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average [2, 8–10]. Mapping >100 s of crossovers at hotspot scale (kb) can be challenging, due to the low number of total crossovers per meiosis. For example, measurement of crossover hotspots in *Arabidopsis* and maize has shown hotspot genetic map lengths that are close to 0.1 cM (or 0.1 % recombination) [9, 11–13]. Therefore, to see >100 crossovers at such a hotspot requires screening of at least 100,000 meioses. For these reasons, specific methods are required for quantification and sequencing of crossover molecules at the physical scale of hotspots.

One approach to study crossover hotspots is to screen very high numbers of meioses by isolating gamete DNA from individuals that are heterozygous in the hotspot region of interest (Fig. 1). In humans and mice this has primarily been achieved using sperm [14–20], but eggs have also been analyzed in mice [21, 22]. In *Arabidopsis*, an equivalent approach uses genomic DNA isolated from pollen (multicellular haploid gametophytes) [11–13, 23, 24]. These methods rely on recombinant crossover and non-recombinant parental molecules being distinguishable by allele-specific PCR amplification (Fig. 1). Titration can be used to estimate the relative concentrations of parental to recombinant molecules, in order to measure crossover frequency (Fig. 1) [18, 23]. Amplified single crossover molecules can then be internally Sanger sequenced, or otherwise genotyped, to identify recombination sites to the resolution of individual polymorphisms (Fig. 1) [18, 23]. Varying allele-specific oligonucleotide (ASO) and non-allele-specific universal oligonucleotide (UO) configurations can also be used to detect non-crossovers and gene conversion events [18, 23, 25]. Together, these methods allow high-resolution studies of recombination frequency and topology within individual crossover hotspots.

Here, we report detailed methodologies for pollen typing that allow the study of crossover hotspots in plant genomes. First, we describe a simplified and robust allele-specific PCR amplification method that is highly sensitive. We provide information on critical steps including pollen genomic DNA extraction, choice of target hotspot locations and allele-specific oligonucleotide (ASO) design. Next, we describe a pollen typing qPCR technique that allows rapid estimation of hotspot crossover frequency, without the need

Fig. 1 (continued) amplification (Subheading 3.6, **steps 10–14**). (**e**) Crossover and parental molecules are quantified using titration (Subheading 3.6). (**f**) Single crossover molecule amplification products are Sanger sequenced to identify internal recombination sites (Subheading 3.7). (**g**) Quantitative PCR analysis of crossover frequency, following the first round of allele-specific PCR amplification (Subheading 3.8). These data are for the *3a* hotspot and are shown in Table 4 [12]. (**h**) Mass amplification and high-throughput sequencing of crossover molecules (Subheading 3.9). (**i**) Identification of crossovers using paired-end sequencing reads (Subheadings 3.9 and 3.10)

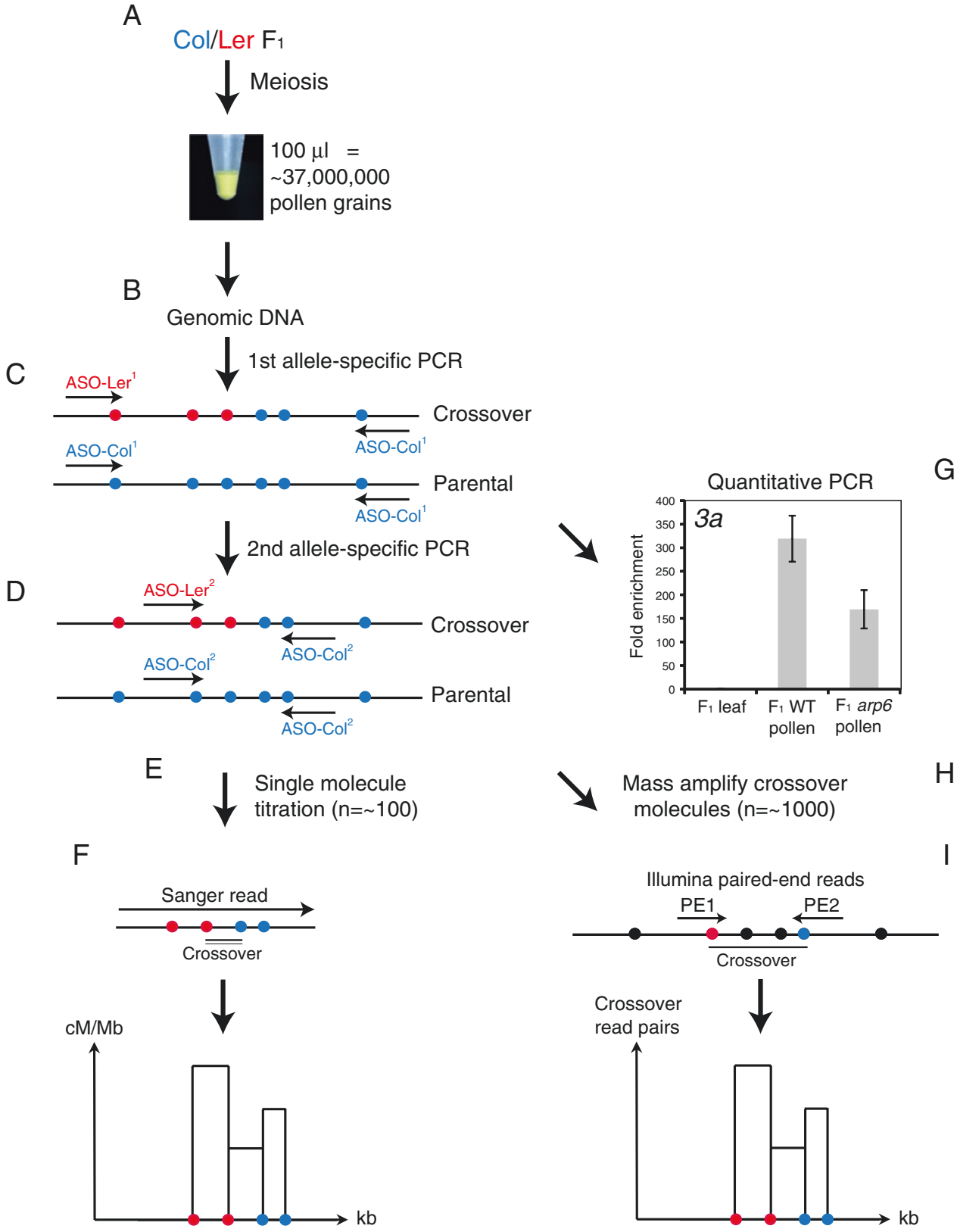

Fig. 1 Diagram illustrating pollen typing methods for crossover hotspot analysis. (**a**) Purification of pollen grains (Subheading 3.1). 100 µl of pollen suspension is shown, which is equivalent to approximately 37,000,000 pollen grains. (**b**) Genomic DNA extraction from pollen (Subheading 3.2). (**c**) 1st round of allele-specific PCR using oligonucleotides specific for Ler (*red*) or Col (*blue*) templates. ASO configurations for crossover or parental molecule amplifications are indicated. Polymorphic sites are represented by *colored circles*. (Subheading 3.6, **steps 1**–**9**). (**d**) 2nd allele-specific PCR as for (C), but using a nested set of ASOs for

for extensive titration experiments (Fig. 1). Finally, we describe a new method to convert large numbers of amplified crossover molecules into high-throughput sequencing libraries, enabling deep sampling of recombination events within hotspots (Fig. 1).

2 Material

2.1 Pollen Purification and DNA Extraction

1. Blender (Elecheck).
2. 80 μm nylon mesh (Normesh).
3. 1 mm glass beads (Sigma-Aldrich Z273619).
4. 3 mm glass beads (Sigma-Aldrich Z143928).
5. 10% sucrose.
6. Lysis buffer: 100 mM NaCl, 50 mM Tris–HCl (pH 8), 1 mM EDTA, 1% SDS. Add dithiothreitol (DTT) to 1 mM immediately prior to use.
7. Proteinase K (20 mg/ml) (Melford MB2005).
8. Liquid phenol, saturated with 1 M Tris–HCl (pH 8).
9. Chloroform–isoamyl alcohol (24:1 volume:volume).
10. Isopropanol.
11. 70% ethanol.
12. TE buffer: 10 mM Tris–HCl (pH 8), 1 mM EDTA.

2.2 DNA Purification

1. DNase-free RNase A (10 mg/ml) (Qiagen 19101).
2. 3 M sodium acetate (pH 5.2).
3. 100% ethanol.
4. 70% ethanol.
5. TE buffer: 10 mM Tris–HCl (pH 8), 1 mM EDTA.
6. Qubit dsDNA BR Assay Kit (Invitrogen, Q32850).
7. Qubit fluorometer (Invitrogen).

2.3 Allele-Specific PCR

1. TaKaRa Ex Taq DNA Polymerase (TaKaRa, RR001C).
2. 10× Ex Taq buffer (TaKaRa, RR001C).
3. dNTP, 2.5 mM (TaKaRa, RR001C).
4. Nuclease-free W=water (Ambion, AM9937).
5. 96-well PCR plates (STARLAB, E1403-100).
6. 96-well PCR plate seals (Thermo Scientific, AB0558).
7. 96-well plate centrifuge (Labnet).
8. 96-well PCR thermal cycler (Bio-Rad Tetrad2).
9. Agarose (Melford, MB1200).

10. Ethidium bromide.
11. HyperLadder 1 kb (Bioline, H1-415106).
12. 5× DNA loading buffer (Bioline, BIO37045).

2.4 Mapping Crossover Sites by Sanger Sequencing

1. Exonuclease (EXOI, New England Biolabs, M0293L 20 U/µl).
2. Shrimp alkaline phosphatase (SAP, Takara, 2660A 1U/µl).
3. BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).
4. 5× BigDye sequencing buffer (Applied Biosystems).

2.5 Pollen Typing qPCR

1. 10× SYBR green (Sigma-Aldrich, S9430).
2. 10× DNA Polymerase PCR Buffer (200 mM Tris–HCl (pH 8.4), 500 mM KCl, 15 mM $MgCl_2$).
3. GoTaq DNA Polymerase (Promega, M3001).
4. Real time PCR thermal cycler (Bio-Rad CFX96).

2.6 Pollen Typing Sequencing Library Construction

1. QIAquick Gel Extraction Kit (Qiagen, 28704).
2. Bioruptor standard (Diagenode).
3. TruSeq DNA Sample prep kit v2 (Illumina).

3 Methods

3.1 DNA Extraction

1. Collect whole *Arabidopsis thaliana* inflorescences and immerse them in ice-cold 10% sucrose. For example, 20 ml 10% sucrose is typically used for one densely packed 50 ml Falcon tube of collected inflorescences. The submerged material can be frozen at −20 °C, or used directly in **step 2**.
2. Disrupt inflorescences using a blender with 3×2 s pulses at maximum speed. Pollen grain walls will remain intact after this treatment.
3. Filter the tissue homogenate through 80 µm nylon mesh into 50 ml Falcon tubes. If multiple tubes are collected, the pollen can be combined following **step 5**.
4. Centrifuge the filtrate at 350×*g* for 10 min at 4 °C.
5. Discard the supernatant and resuspend the pellet, which contains pollen grains and tissues fragments, in 30 ml of ice-cold 10% sucrose.
6. Centrifuge the resuspended pellet at 100×*g* for 10 min at 4 °C.
7. Discard the supernatant and resuspend the pellet in 30 ml of ice-cold 10% sucrose.
8. Centrifuge the resuspended pellet at 100×*g* for 3 min at 4 °C.

9. Repeat **steps 7** and **8**. This process should be repeated until sufficient pollen for downstream experiments has been collected.
10. By this step, a bright yellow pellet enriched for pollen should be obvious (Fig. 1a). Remove the supernatant and resuspend the remaining pollen pellet/suspension in 100 µl aliquots into 1.5 ml eppendorf tubes. From cell counting experiments we estimate that 100 µl contains approximately 37,000,000 pollen grains (Fig. 1a). Pollen aliquots can be stored at −20 °C, or used directly in **step 11**.
11. Resuspend the pollen grain pellet in 4 vol of Lysis Buffer, containing freshly added dithiothreitol (DTT).
12. Add proteinase K to a final concentration of 20 µg/ml.
13. Incubate overnight at 65 °C. Following overnight digestion, the color of the pollen suspension will change from yellow to brown.
14. Add 200 µl of 1 mm glass beads and 4 × 3 mm glass beads.
15. Vortex at full speed for 6 min (*see* **Note 1**).
16. Centrifuge at 17000 × *g* for 5 min.
17. Transfer the supernatant (400 µl) to a new tube containing 100 µl of Lysis Buffer (=A tube).
18. Add 500 µl of fresh Lysis Buffer without SDS to the bead pellet (=B tube).
19. Vortex the B tube at full speed for 3 min.
20. Add 1 volume (500 µl) of phenol saturated with 1 M Tris–HCl (pH 8) to each of the A and B tubes, followed by mixing by hand inversion.
21. Gently mix for 30 min on a rocking wheel.
22. Centrifuge at 15,000 × *g* for 10 min.
23. Carefully transfer the supernatant to a new Eppendorf tube.
24. Add an equal volume of chloroform–isoamyl alcohol (24:1 volume:volume). Homogenize by gentle hand shaking.
25. Centrifuge at 15,000 × *g* for 10 min.
26. Carefully transfer the supernatant to a new Eppendorf tube.
27. Add 0.7 vol of isopropanol. Incubate for 10 min at room temperature.
28. Centrifuge at 15,000 × *g* for 10 min at 4 °C. Discard the supernatant.
29. Wash the pellet with 1 ml of 70% ethanol.
30. Centrifuge at 15,000 × *g* for 5 min at 4 °C. Discard the supernatant.

31. Repeat **steps 29** and **30**.
32. Allow the pellet to air-dry at room temperature for 5 min. Do not overdry the DNA pellet, which will make it difficult to redissolve.
33. Carefully resuspend each pellet in 100 µl of TE buffer and combine the DNA from the A and B tubes (*see* **Note 2**).

3.2 DNA Purification

1. Add RNaseA to a final concentration of 100 µg/ml.
2. Incubate for 30 min at room temperature.
3. Add 0.1 volume of 3 M sodium acetate. Homogenize by gentle hand shaking.
4. Add 2.5 volumes of 100% ethanol. Homogenize by gentle hand shaking.
5. Incubate on ice for at least 30 min.
6. Centrifuge at 15,000 × *g* for 20 min at 4 °C.
7. Wash the pellet with 1 ml of 70% ethanol.
8. Centrifuge at 15,000 × *g* for 5 min at 4 °C. Discard the supernatant.
9. Repeat **steps 7** and **8**.
10. Allow the pellet to air-dry at room temperature for 5 min. Do not overdry the DNA pellet, which will make it difficult to redissolve.
11. Carefully resuspend the pellet in 200 µl of TE buffer.
12. Repeat **steps 3–10**.
13. Carefully resuspend the pellet in 100 µl of TE buffer (*see* **Note 2**).
14. Quantify DNA concentrations using a fluorometer. DNA quality should also be assessed using 1% agarose gel electrophoresis and ethidium bromide staining, which is added to a final concentration of 0.5 µg/ml (Fig. 2).
15. DNA is then either used directly for pollen typing (Subheadings 3.6–3.9) or frozen in 5–20 µl aliquots.

3.3 Identification of Candidate Crossover Hotspots for Pollen Typing

The design and validation of hotspot allele-specific oligonucleotides (ASOs) represents a considerable amount of work, and so it is important to carefully select hotspots before the start of the experiment. A variety of methods, data and technical considerations are important in this selection procedure. First, an attempt should be made to identify regions that show evidence for high crossover levels using more than one kind of evidence. Hotspots can be identified using population genetics approaches that analyze patterns of linkage disequilibrium, to provide evidence of historical crossovers [19, 26, 27]. This kind of evidence relies on the

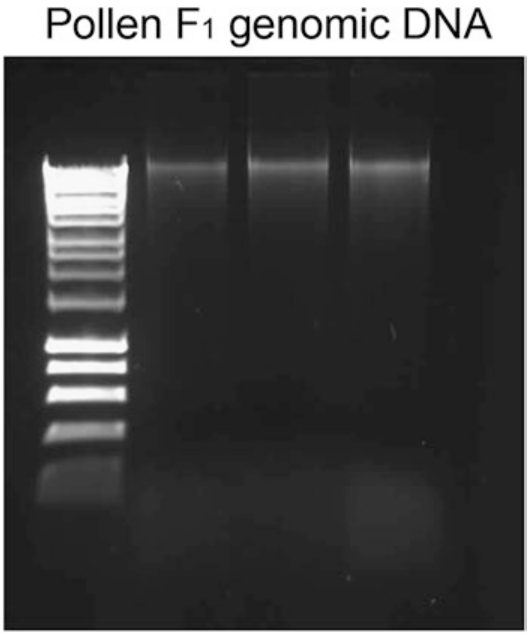

Fig. 2 Gel electrophoresis analysis of pollen genomic DNA. *Arabidopsis* pollen genomic DNA separated by electrophoresis using a 1 % agarose gel containing ethidium bromide. The *three lanes* show 1 μl of DNA, with the concentrations of 34.4, 42.1 and 53.2 ng/μl

availability of high quality natural polymorphism datasets, such as the 1001 *Arabidopsis* Genomes Project [28]. Hotspots can be inferred from these datasets using a variety of statistical methods, which estimate the population-scaled recombination rate *4Ner*, where *Ne* is the effective population size and *r* is the per generation crossover rate [26, 27, 29, 30]. While hotspots estimated via these approaches have been successfully predicted and validated using pollen typing [11, 12], they have important caveats. These approaches use recombination estimated over many haplotype combinations from both male and female meiosis. Therefore, predicted hotspots may not occur in a specific cross (e.g., Col-0 × Ler-0), for example if structural heterozygosity exists at the hotspot, or if hotspot activity is specific to female meiosis.

Another means of identifying high recombination regions is by using existing large datasets of experimentally mapped crossovers [31–35]. These datasets are typically not deep enough to allow prediction of fine-scale (kb) hotspot locations. However, the likely hotspot region can be identified using additional information. Plant hotspots are known to occur primarily at gene promoters and terminators [9, 12, 36–39], so gene annotation can be used to predict likely hotspot locations within high recombination regions. Additionally, low nucleosome density in promoters, and high H3K4^{me3} and H2A.Z within genes have been associated with plant crossover hotspots, which can be identified via analysis of available epigenomic datasets [12, 40–43]. Finally, repetitive sequences in

plant genomes, such as densely DNA methylated transposons, are generally silent for recombination [11, 24, 44]. Hence, heterochromatin and repetitive regions should be avoided for pollen typing analysis, unless strong prior evidence for crossovers exists and structural polymorphism is well understood.

Once candidate hotspot regions have been identified, it is critically important to have accurate knowledge of polymorphisms in this region for the two accessions to be crossed (e.g., Col-0 and Ler-0). It is recommended that target hotspot regions are PCR amplified and Sanger sequenced from the laboratory parent lines to be used for crossing, in order to identify all insertions/deletions (indels) and single nucleotide polymorphisms (SNPs), which are required for allele-specific PCR amplification. It may also be informative to perform Southern blotting and hybridization in order to understand structural variation, which can inhibit recombination [15, 16, 37].

3.4 Allele-Specific Oligonucleotide (ASO) Design

Two nested pairs of ASOs are required to PCR-amplify, quantify, and sequence crossover molecules [18, 23] (Fig. 1). This key step must be carefully performed and the specificity and efficiency of ASOs optimized. To test ASO specificity and efficiency, the primer pairs are combined with non-allele-specific universal oligonucleotides (UOs) and used to amplify from parental DNA templates separately (e.g., Col-0 and Ler-0). ASOs are considered unsuccessful if multiple amplification bands are observed, or poor template specificity is seen using the parent accession template DNA. Examples of successful ASOs used for pollen typing in *Arabidopsis* are shown in Table 1. Figure 3 shows the results of test amplifications with successful (e.g., ASO1, 2, and 6) and unsuccessful (e.g., ASO3, 4, 5, 7–11) ASOs. The successful ASOs 1, 2 and 6 correspond to KC417, KC418, and KC465 (Table 1). Finally, pairs of optimized ASOs must then amplify crossovers specifically from F_1 pollen DNA and not F_1 leaf DNA (Fig. 4).

3.4.1 Length of Amplicons

ASOs should be designed to anneal to sequences flanking candidate hotspot regions. Our protocol allows amplicons between 6 and 10 kb in length to be routinely analyzed from pollen genomic DNA. The analysis described in Subheading 3.3 should be used to predict suitable locations for pollen typing.

3.4.2 Allele-Specific Oligonucleotide Selection

ASOs must strongly discriminate between the polymorphic parental templates during PCR amplification (Fig. 3). ASOs should be designed such that their 3′-ends anneal to polymorphic sites, where mismatches will have the greatest effect on the extension of the probe (Table 1). ASOs to be used as pairs in crossover analysis need to amplify equally efficiently from their specific template, at a given melting temperature (T_m) (Fig. 3). The following features should be considered during ASO design.

Table 1
Sequences of pollen typing allele-specific and universal oligonucleotides

Hotspot	ASO/UO	Name	Sequence (5′-3′)	Length	GC %	Mismatch	Poly-dC
3a	ASO	6339—ColF 1st	GAGAAACCAACCACTTCT	18	44	–	–
3a	ASO	6401—LerR 1st	CAAGGATTCTCTATATCTAC	20	35	–	–
3a	ASO	6401—ColR 1st	GGGGATTCTCTATATCTAGGA	21	43	–	–
3a	ASO	6341—ColF 2nd	CCCCCTTTCAAATTGATACAACAA	24	38	–	Yes
3a	ASO	6399—LerR 2nd	CCCAAGTTTTCTTCTCAAGCCA	22	45	Yes	Yes
3a	ASO	6399 – ColR 2nd	CCCAAGTTTTCTTCTCAAGCCT	22	45	Yes	Yes
3a	UO	3a-UF	GACGCTAGGCGCTGGTAAG	19	63	–	–
3a	UO	3a-UR	CTCGACCGGGGTACACCATC	20	65	–	–
3b	ASO	KC156—LerF 1st	AGAAAATAGATAACTCTTCG	20	30	–	–
3b	ASO	KC167—ColF 1st	CTTCTTGATTCACACCTTA	19	37	–	–
3b	ASO	KC166—ColR 1st	ATCGAATTCGCGACTAG	17	47	Yes	–
3b	ASO	KC160—LerF 2nd	CCCACTAAGACACAAATAC	19	42	–	Yes
3b	ASO	KC168—ColF 2nd	AATGTAATGCTCTGCTCC	18	44	–	–
3b	ASO	KC152—ColR 2nd	GCTTTGAAAATTCTTGTC	18	33	Yes	–
3b	UO	3b-UF	GCGGTACACCTCATGTCTAC	20	55	–	–
3b	UO	3b-UR	GGATTCCGCTGCTTCAAGTC	20	55	–	–
RAC1	ASO	KC459—LerF 1st	CTGACTTGAGTGATCGCAA	19	47	–	–
RAC1	ASO	KC493—ColF 1st	AAAACGTGCAACCTAAGAAC	20	40	–	–

RAC1	ASO	KC418—ColR 1st	ATTTCACCCGATGTAGTCC	19	47	–	–
RAC1	ASO	KC465—LerF 2nd	GTGGCCGCAAGCAAAAATAT	20	45	–	–
RAC1	ASO	KC495—ColF 2nd	AACAGATTGGTCTCATTG	18	39	–	–
RAC1	ASO	KC417—ColR 2nd	TAGTTTTTCTGACCCCAC	18	44	–	–
RAC1	UO	RAC1-UF	CTACAAAGCCATATAGCCAGAGCC	24	50	–	–
RAC1	UO	RAC1-UR	GATTCACTAAAGGAGAGCCAAAGTT	25	40	–	–

These ASOs have been successfully used to analyze crossover hotspots by pollen typing in *Arabidopsis* [11, 12, 24]. The relevant Col-0/Ler-0 polymorphic positions are highlighted in ASOs in *red*. *Blue* indicates universal mismatches added to improve amplification specificity. *Green* cytosines (Poly-dC) were added to the 5′ end of primers to increase amplification efficiency

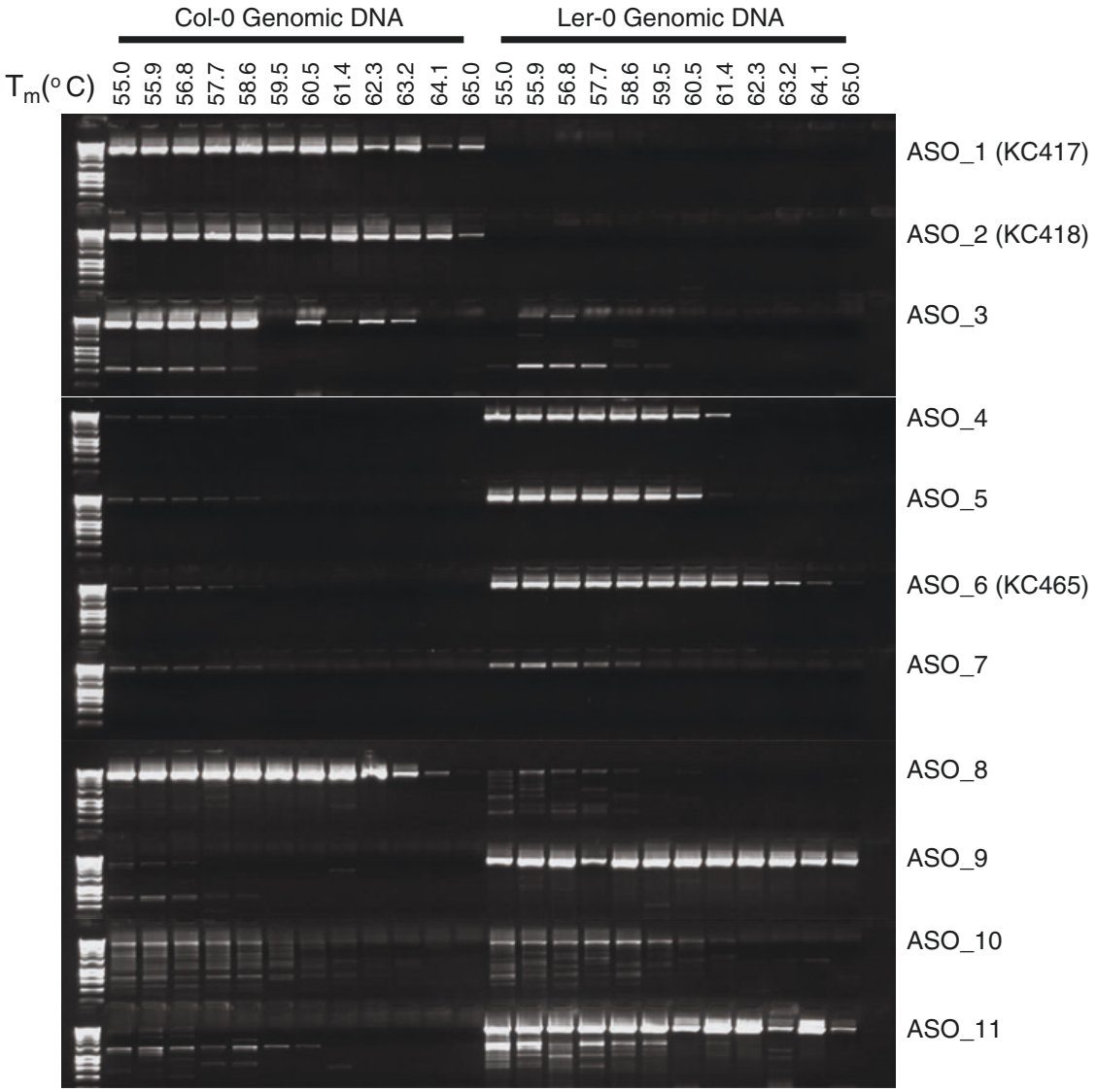

Fig. 3 Optimizing allele-specific PCR amplification. PCR amplification products from ASO optimization experiments were separated by electrophoresis using a 1 % agarose gel containing ethidium bromide. Each ASO was used with a second universal oligonucleotide (UO) at a range of melting temperatures, with either Col-0 or Ler-0 genomic DNA templates. ASO_1 and ASO_2 are successful, showing high template specificity (Table 1). ASO_6 is also successful, though only when 63 °C is used as an annealing temperature (Tables 1 and 2). ASO_3, 4, 5, 8, 9, 10 and 11 are not suitable for pollen typing, due to poor template specificity or amplification of non-specific products

1. Design ASOs to anneal to polymorphic indel sequences between 5 and 1,000 base pairs in length, located in non-repetitive sequence.
2. Alternatively, if no suitable indels are available, identify a site with multiple adjacent SNPs.
3. Design ASOs with 30–50 % GC content, a length between 16 and 21 nucleotides (nt) and an estimated T_m between 57 and 62 °C. These parameters can be calculated using websites such as http://www.basic.northwestern.edu/biotools/

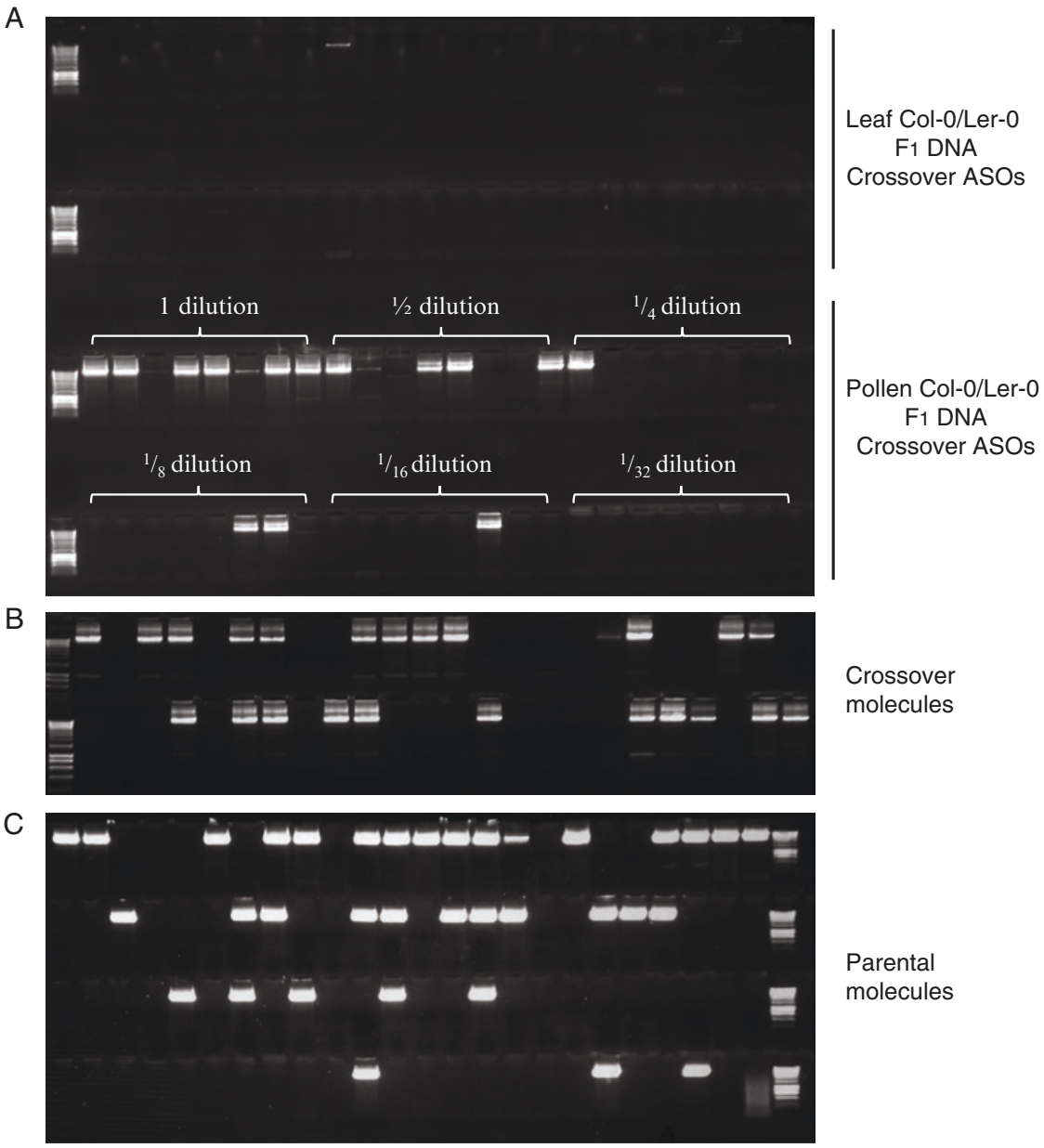

Fig. 4 Amplification and titration of single crossover and parental molecules from pollen genomic DNA. (**a**) Crossover PCR amplification products using nested pairs of ASOs were separated by gel electrophoresis using an agarose gel containing ethidium bromide. Amplification of crossover molecules is evident from Col-0/Ler-0 F_1 pollen genomic DNA, but not Col-0/Ler-0 F_1 leaf genomic DNA. 8 reactions were performed for each DNA dilution analyzed (Subheading 3.6, **steps 1–16**). (**b**) Representative 1 % agarose-ethidium bromide gel showing single crossover amplifications from 48 reactions, where approximately half (25 of 48) are negative (Subheading 3.6, **step 29**). (**c**) Representative 1 % agarose-ethidium bromide gel showing single parental molecule amplifications in a dilution series of 24 reactions. 6.5 μl of a 1:2000 dilution of 10 ng/μl pollen DNA solution was added to a PCR master mix. A dilution series of 1:1, 1:2 and 1:4 were made as described (Subheading 3.6, **step 34**). 24 PCR reactions were analyzed for each dilution

OligoCalc.html. Shorter ASOs (e.g., 17 nt) should have higher GC content (e.g., 45 %) and longer ASOs (e.g., 20 nt) should have a lower GC (30 %), in order to optimize annealing temperature.

4. Avoid ASOs predicted to form dimers or secondary structures, which can also be identified using the above oligo calculation website. Additional mismatches to the template can be added to ASOs to mitigate primer dimer formation and secondary structure, as required.
5. An additional mismatch (e.g., underlined bold T) close to the ASO 3′ end (-2 or -3 position) can be added in order to increase ASO specificity (e.g., ATCGAATTCGCGAC**T**A**G**). This can be useful for short ASOs with high GC content.
6. 3-6 poly-dC (e.g., underlined CCC) or poly-dG can optionally be added to the 5′-end of ASOs (e.g., **CCC**ACTAAGACACAAATA**C**). This increases annealing temperature and can improve amplification efficiency, which is particularly useful for short ASOs with low GC content.

3.5 Optimisation of ASO Specificity and Efficiency

It is critical to experimentally test ASO template specificity and amplification efficiency using gradient PCR. These steps should be repeated until at least two nested pairs of high quality ASOs are obtained per hotspot (*see* Introduction to Subheading 3.4) (Fig. 1). For these assays universal, non-allele-specific oligonucleotides (UO) should also be designed in positions close to the ASOs. UOs should be designed with annealing temperatures above 65 °C, such that the T_m of the ASO has the greater effect on amplification efficiency.

1. Dilute leaf genomic DNA for the two *Arabidopsis thaliana* accessions (e.g., homozygous Col-0 and homozygous Ler-0), which are to be used in pollen typing analysis, to 5 ng/μl with TE.
2. Prepare a cocktail for 13 PCR reactions in a 1.5 ml Eppendorf tube, in order to test a single ASO using gradient PCR in 96-well plate, using DNA from the first accession (e.g., Col-0), as described below.

Components	Volume (μl)	13 Reactions
Genomic DNA (5 ng/μl)	0.2	2.6
10× Ex Taq buffer	1	13
dNTP, each 2.5 mM	1	13
ASO (5 μM)	1	13
UO (universal oligo) (5 μM)	1	13
Ex Taq (5 U/μl)	0.05	0.65
Nuclease-free water	5.75	74.75
Total	10	130

3. Prepare an additional 13 PCR reactions to test the same ASO with genomic DNA from the other *Arabidopsis thaliana* accession (e.g., Ler-0).
4. Transfer 10 µl of the reaction cocktail to each of 24 wells (e.g., A1-A12 for Col-0, B1-B12 for Ler-0) in a 96-well plate, using an automatic pipette.
5. Run a gradient PCR in a thermal cycler using the following parameters:

   1 cycle of,

   94 °C, 2 min;

   30 cycles of,

   94 °C, 30 s,

   55–65 °C (gradient), 30 s,

   68 °C (1 min/kb);

   1 cycle of 68 °C, 1 min/kb +1 min

   Hold at 4 °C.
6. Add 2.5 µl of 5× DNA loading buffer to each well and analyze the PCR products using gel electrophoresis with a 1.2% agarose gel and ethidium bromide staining (Fig. 3).
7. Assess whether ASOs are able to amplify PCR products efficiently and specifically from one parental template (e.g., Col-0) and not the other (e.g., Ler-0) (Fig. 3). A successful ASO will amplify a single band only from one parental template.
8. For ASOs that show PCR amplification specificity at higher temperatures (e.g., 59–65 °C), but produce weak or non-specific PCR bands at lower temperatures (e.g., 55–58 °C), further optimisation is required by either changing ASO length, adding mismatches and/or poly-dC (*see* Subheading 3.4.2), or redesigning ASOs at an alternative location.

3.6 Titration of Single Crossover and Parental Molecules

In order to estimate the recombination rate of a given hotspot it is necessary to estimate the concentration of crossover (recombinant) and parental (non-recombinant) molecules via titration (Fig. 4) [18, 23].

1. Prepare the first reaction cocktail using F_1 pollen genomic DNA (e.g., Col-0/Ler-0 F_1) for 20 PCR reactions and mix well by pipetting ten times. Depending on the amount of pollen extracted from, the concentration of F_1 pollen genomic DNA is typically between 10 and 50 ng/µl (*see* **Note 2**).
2. Prepare a second reaction cocktail without DNA template sufficient for 50 reactions and transfer 100 µl to each of five 1.5 ml tubes (1st, 2nd, 3rd, 4th, 5th), which will be used for serial twofold dilutions.

Components	Volume (μl)	20 Reactions	50 Reactions
Template	–	–	0
10× Ex Taq buffer	1	20	50
dNTP, each 2.5 mM	1	20	50
ASO 1st F (5 μM)	1	20	50
ASO 1st R (5 μM)	1	20	50
Ex Taq (5 U/μl)	0.05	1	2.5
Nuclease-free water	5.95	–	297.5
Total	10	200	Each 100 μl

3. Transfer 100 μl from the first reaction cocktail to the 1st dilution tube and mix in order to achieve a twofold dilution.
4. Transfer 100 μl from the 1st dilution tube to the 2nd dilution tube and mix well to achieve a twofold dilution.
5. In the same way dilute into the 3rd, 4th, and 5th tubes in order to achieve 8-, 16- and 32-fold dilutions, by transferring 100 μl from the 2nd, 3rd, and 4th dilution tubes respectively.
6. Transfer 10 μl from first reaction cocktail to eight wells (e.g., A1-H1) of a 96-well plate.
7. Transfer 10 μl from the 1st, 2nd, 3rd, and 4th dilution tubes to eight wells each (e.g., A2-H2, A3-H3, A4-H4, A5-H5 and A6-H6).
8. Repeat **steps 1–7**, but using the same amount of Col/Ler F_1 leaf genomic DNA in the remaining wells (e.g., A7-H12). In this DNA sample both parental allelic templates are present, but crossovers should be absent. This serves as a negative control for PCR artifacts, including template switching.
9. Run the first round of touchdown PCR in a thermal cycler using the following parameters (*see* **Note 3**):

   1 cycle of,

   94 °C for 2 min 30 s;

   8 cycles of,

   94 °C for 30 s, 4 °C higher than ASO annealing temperature (decrease by 0.5 °C per cycle), for 45 s,

   68 °C for 1 min/kb + 30 s;

   22–25 cycles of,

   94 °C for 20 s,

   ASO annealing temperature (°C) for 30 s,

   68 °C for 1 min/kb + 30 s;

1 cycle of,

68 °C 1 min/kb + 1 min;

Hold at 4 °C.

10. Dilute the PCR products 20-fold by adding 190 µl of 10 mM Tris–HCl (pH 8) to each well.
11. Prepare a reaction cocktail for a 96-well PCR plate for the second round of ASO PCR, as below.

Components	Volume (µl)	100 Reactions
Diluted first PCR product	1	Each
10× Ex Taq buffer	1	100
dNTP, each 2.5 mM	1	100
2nd ASO F (5 µM)	1	100
2nd ASO R (5 µM)	1	100
Ex Taq (5 U/µl)	0.05	5
Nuclease-free water	4.95	495
Total	10	Each 9

12. Aliquot 9 µl of the reaction cocktail to each well using an automatic pipette.
13. Transfer 1 µl of the diluted 1st round PCR plate to each well of the 2nd round PCR plate using an eight channel pipette.00
14. Run the second round of touchdown PCR in a thermal cycler using the following parameters:

    1 cycle of,

    94 °C for 2 min 30 s;

    8 cycles of 94 °C for 30 s,

    04 °C higher than ASO annealing temperature (decrease by 0.5 °C per cycle) for 45 s,

    68 °C for 1 min/kb + 30 s;

    22–25 cycles of,

    94 °C for 20 s,

    ASO annealing temperature (°C) for 30 s,

    68 °C for 1 min/kb + 30 s,

    1 cycle of,

    68 °C 1 min/kb + 1 min,

    Hold at 4 °C.
15. Add 2.5 µl of 5× DNA loading buffer to each well and analyze the PCR products by gel electrophoresis using a 1.2% agarose gel and ethidium bromide staining (Fig. 4).

16. Repeat **steps 1–15** to test different ASO combinations and optimize annealing temperatures and cycle numbers in the first and second rounds of PCR. Repeat until strong amplification bands appear specifically when F_1 pollen genomic DNAs are used as a template, but which are absent when leaf genomic DNA is used (Fig. 4a). Optimizing this step is critical for the analysis of single crossover molecules (*see* **Note 4**). ASOs and PCR parameters are listed in Table 2 for analysis of different *Arabidopsis* hotspots.
17. Estimate the concentration of amplifiable crossover molecules, based on the dilution series. Identify a DNA concentration where approximately half of the reactions are negative and half are positive (Fig. 4a). The concentration of crossover molecules (c1) can be estimated as follows.

$$c1 = -\ln(N / T) / V,$$

where N is the number of negative reactions at a given dilution,

T is the total number of reactions at a given dilution,

V is the pollen DNA volume (μl) per reaction, and

ln is the natural logarithm.

For example, consider a case in which the 3rd dilution of eight wells (e.g., A4-H4) had four positive and four negative wells and 0.14 μl of input DNA was used for each undiluted reaction. In the 3rd dilution wells, there is 0.0175 μl of template DNA. Therefore, $c1 = -\ln(4/8)/(0.14/8) = 39.61$ crossovers/μl. If the 4th dilution of the same DNA had 1 positive band, then $c1 = -\ln(7/8)/(0.14/32) = 30.52$ crossovers/μl. Therefore, the input pollen DNA has roughly 35.07 crossovers/μl. Increasingly accurate estimates of crossover concentrations should then be determined by increasing the number of reactions with varying amounts of input DNA, as described below.

18. Estimate the volume of input DNA that produces approximately half negative wells from multiple independent PCR reactions (e.g., 24–96 wells), using the preliminary estimation of crossover concentration (c1) from **step 17**. The volume of DNA input in each reaction is $= -\ln(0.5) \times (1/c1)$. For example, if c1 from eight reactions is 32.88 crossovers/μl, then add $-\ln 0.5 \times (1/32.88)$, or 0.021 μl, to each of the 24 reactions.

Prepare four PCR reaction cocktails (a-d), each for 25 reactions, with varying DNA template input amounts, as below.

Table 2
Pollen typing PCR parameters for analysis of *Arabidopsis* hotspots

Hotspot	Molecules	First PCR	Annealing temperature	Second PCR	Annealing temperature
3a	Parental	6339—Col F 1st 6401—Col R 1st	59–55 °C (−0.5 °C/cycle)	6341—Col F 2nd 6399 – Col R 2nd	59–55 °C (−0.5 °C/cycle)
3a	Crossover	6339—Col F 1st 6401—Ler R 1st	59–55 °C (−0.5 °C/cycle)	6341—Col F 2nd 6399—Ler R 2nd	59–55 °C (−0.5 °C/cycle)
3b	Parental	KC167—Col F 1st KC166—Col R 1st	59–55 °C (−0.5 °C/cycle)	KC168—Col F 2nd KC152—Col R 2nd	57–53 °C (−0.5 °C/cycle)
3b	Crossover	KC156—Ler F 1st KC166—Col R 1st	59–55 °C (−0.5 °C/cycle)	KC160—Ler F 2nd KC152—Col R 2nd	57–53 °C (−0.5 °C/cycle)
RAC1	Parental	KC493—Col F 1st KC418—Col R 1st	65–63 °C (−0.4 °C/cycle)	KC495—Col F 2nd KC417—Col R 2nd	65–63 °C (−0.4 °C/cycle)
RAC1	Crossover	KC459—Ler F 1st KC418—Col R 1st	65–63 °C (−0.4 °C/cycle)	KC465—Ler F 2nd KC417—Col R 2nd	65–63 °C (−0.4 °C/cycle)

Components	Volume (μl)	25 Reactions
DNA template (*see* (a)–(d) below)	–	–
10× Ex Taq buffer	1	25
dNTP, each 2.5 mM	1	25
ASO 1st F (5 μM)	1	25
ASO 1st R (5 μM)	1	25
Ex Taq (5 U/μl)	0.05	1.25
Nuclease-free water	5.95	–
Total	10	250

(a) 1st reaction cocktail with volume of DNA template input according to **steps 17** and **18**

(b) 2nd reaction cocktail with $^1/_2$ DNA input volume of 1st reaction cocktail

(c) 3rd reaction cocktail with $^1/_4$ DNA input volume of 1st reaction cocktail

(d) 4th reaction cocktail with $^1/_8$ DNA input volume of 1st reaction cocktail

19. Transfer 10 μl from the 1st, 2nd, 3rd, 4th reaction cocktails to 24 wells (e.g., A1-H3, A4-H6, A7-H9 and A10-H12 respectively) of a 96-well plate.
20. Run the first round of allele-specific touchdown PCR (Table 2), as in **step 9**.
21. Dilute the PCR product from **step 21**, 20-fold by adding 190 μl of 10 mM Tris–HCl (pH 8) to each well.
22. Prepare a reaction cocktail for a 96-well PCR plate for the second round of allele-specific PCR, as in **step 11**.
23. Aliquot 9 μl of the reaction cocktail to each well, using an automatic pipette.
24. Transfer 1 μl of the diluted 1st round PCR plate (**step 22**) to each well of the 2nd round PCR plate, using an eight channel pipette.
25. Run the second round of allele-specific touchdown PCR as in **step 14**.
26. Add 2.5 μl of 5× DNA loading buffer to each well and analyze the PCR products using gel electrophoresis with a 1.2% agarose gel and ethidium bromide staining. Score the number of positive and negative amplifications (*see* **Note 4**).
27. Estimate the concentration (c2) of amplifiable crossover molecules, based on the dilution series of 24 reactions, as for **step 17**. For example, if there are 12 positive PCR bands in

the first set of 24 wells, when 0.019 μl input DNA was used for each reaction, $c2 = -\ln(12/24)/0.019 = 36.48$ crossovers/μl. If there were eight positive PCR bands in the second set of reactions, when 0.0095 μl input DNA were used for each reaction, $c2 = -\ln(16/24)/0.0095 = 42.68$ crossovers/μl. Here the mean c2 is 39.58 crossovers/μl.

28. Repeat **steps 18–27** with two sets of 48 reactions. For the first set, add the amount of input DNA which generates about half positive and half negative amplifications to each of the 48 wells, e.g., $-\ln(0.5) \times (1/c2) = 0.018$ μl. For the second set of reaction, use half this volume as input DNA, e.g., 0.009 μl, compared with the first 48 reactions.
29. Estimate the concentration (c3) of crossover molecules as in **step 17**.
30. Finally repeat **steps 18–27** with three sets of 96-well amplifications, using an input amount estimated from c3 to give half positive and half negative reactions.
31. Estimate the concentration (c4) of crossover molecules. The concentrations of crossover molecules from 48 and 3×96 reactions (c3 and c4) should be very similar between independent experiments. Whereas the c1 and c2 estimates from 8 and 24 reactions show greater variation.
32. Determine the final concentration of crossover molecules and standard deviation using the estimates c3 and/or c4 from multiple independent experiments with varying input amounts (Table 3).
33. Given that the genetic length of plant hotspots is typically between 0.1 to 0.5 cM [9, 11–13], the concentration of parental molecules is vastly higher than that of crossover molecules. Therefore, to estimate parental molecule concentrations it is necessary to repeat **steps 1–33** using 500 to 1000-fold diluted F_1 pollen genomic DNA input. Parental amplifications use two pairs of parental ASOs and/or UOs (Fig. 1 and Table 1).
34. Calculate crossover frequency (centiMorgans per megabase, cM/Mb) from the independent titration experiments (**steps 1–34**) using the following formula:

$$\mathrm{cM} = \left(c \,/\, p\right) \times 100$$

c is the concentration of crossover molecules (**step 33**).

p is the concentration of parental molecules (**step 34**).

cM/Mb = cM/(bp of 2nd PCR amplicon/1,000,000)

3.7 Mapping Crossover Sites by Sanger Sequencing

1. Perform first and second rounds of allele-specific PCR in multiple (>3) 96-well plates to amplify single crossover molecules using F_1 pollen genomic DNA as a template. Estimate the amount of DNA template for each reaction

Table 3
Titration of crossover molecules. Average concentration of crossovers from c3a to c4b = 38.4 ± 2.65 crossovers/μl

Estimate	Number of positive wells (P)	Number of negative wells (N)	Number of total wells (T)	μl/well (V)	Crossovers/ μl = −ln((N/T)/V)	Average crossovers/μl	μl for half N/half P
c1a	4	4	8	0.0175	39.61		
c1b	2	6	8	0.00875	32.88	36.24	0.019
c2a	12	12	24	0.019	36.48		
c2b	8	16	24	0.0095	42.68	39.58	0.018
c3a	24	24	48	0.018	38.51		
c3a	15	34	48	0.009	38.32	38.41	0.018
c4a	45	51	96	0.018	35.14		
c4b	30	66	96	0.009	41.63	38.39	0.018

using 1/concentration of crossovers × -ln(0.5). This will lead to PCR amplifications where ~69 % are from single crossover molecules and ~31 % will be from two or more crossovers, 0.5 × ln(0.5)/−0.5 = 0.69. This process should be repeated on a sufficient scale to isolate and sequence 100 or more independent crossovers per hotspot.

2. Add 2.5 µl of 5× DNA loading buffer directly to each well (total 12.5 µl) and run 7 µl of the PCR products using a 1.2 % agarose gel and ethidium bromide staining, in order to identify positive wells (Fig. 4b).
3. Transfer the remaining 5 µl of PCR product from the positive wells to a new 96-well plate.
4. Add 195 µl of 10 mM Tris–HCl (pH 8) to each well.
5. Amplify 1 µl of the diluted PCR products using the same conditions as for the second round of allele-specific PCR (Subheading 3.6, **step 14**).
6. Add 5 µl of the following cocktail (0.3 µl EXO I, 1.3 µl SAP, 3.4 µl D.W) to each well. Exonuclease (EXO) removes single-stranded DNA and shrimp alkaline phosphatase (SAP) dephosphorylates the PCR products to prepare for Sanger sequencing.
7. Incubate at 37 °C for 40 min and then 75 °C for 10 min.
8. Prepare Sanger sequencing reactions by transferring 2 µl of the exonuclease/SAP-treated DNA reactions to 8 µl of the following cocktail in a 96-well plate, or a 0.2 ml PCR tube as below.

Component	Amount per well (µl)
PCR product template	2
5× Sequencing buffer	2
BigDye (Applied Biosystems)	0.9
Primer (10 µM)	1
Nuclease-free water	4.1

9. Run the reactions in a thermal cycler with the following parameters:

   1 cycle of,

   96 °C for 30 s;

   27 cycles of,

   96 °C for 30 s,

   50 °C for 5 s,

   60 °C for 3 min;

   Hold at 4 °C.

10. Sequence the product using an ABI 3700 DNA sequencer, or similar machine, via a service provider.
11. Determine crossover sites by manually checking sequencing data chromatograms using visualization programs such as CLC Main Workbench and Chromas. If the PCR product is derived from two crossover molecules, double peaks will be evident in the chromatograms at polymorphic sites. This allows both crossover locations to be identified. However, if more than two crossover molecules are sequenced in a single reaction it is not generally possible to confidently identify recombination sites.
12. Sequence each crossover PCR product with additional primers until the recombination site is confirmed by observing a change in SNP genotype from one accession to the other (e.g., Col-0->Ler-0). Note this change should match the orientation of the ASOs used in the first and second rounds of allele-specific PCR (Fig. 1). Gene conversion associated with crossovers may also lead to multiple genotype switches in vicinity to a crossover.
13. Calculate the recombination activity in each SNP interval within the amplicon as follows:

$$\text{cM} / \text{Mb} = cM \times (N / T) / (L / 1{,}000{,}000)$$

cM: concentration of crossover molecules/concentration of parental molecules × 100 (Subheading 3.6., **step 35**).

L: Length of interval (bp).

N: Crossover numbers in the interval being considered.

T: Total number of mapped single crossovers.

Representative data for the *3a* hotspot are shown in Table 4.

3.8 Pollen Typing Quantitative PCR

Titration experiments are necessary for accurate hotspot crossover frequency estimation and to isolate single crossover molecules for Sanger sequencing. However, in many cases it is necessary only to estimate hotspot recombination rates during preliminary experiments, or when testing conditions that may influence crossover frequency. For these purposes we describe a pollen typing quantitative PCR (qPCR) assay that is useful to compare relative crossover rates between wild type and mutants, or different environmental conditions (Fig. 1). For these assays the input pollen genomic DNA amount and allele-specific PCR cycle number need to be optimized, according to the hotspot being studied. Crossover and parental amplification products are quantified from pollen and leaf F_1 genomic DNA, which either possess or lack crossovers respectively (Fig. 5).

Table 4
Crossover frequency in each interval within the 3a hotspot. The SNP position (bp) is relative to the TAIR10 Col-0 reference sequence. These data are reproduced from [12]

Chr 3	SNP genotype				
Position	Col-0	Ler-0	Length (bp)	Crossovers	cM/Mb
634,109	A	G	829	11	28.57
634,938	C	T	1,181	1	1.82
636,119	A	C	80	0	0
636,199	T	A	1,084	16	31.78
637,283	T	A	94	2	45.81
637,377	–	A	377	8	45.69
637,754	A	G	220	3	29.36
637,974	A	G	509	22	93.06
638,483	A	T	150	1	14.35
638,633	T	A	6	0	0
638,639	C	T	32	0	0
638,671	C	–	6	0	0
638,677	C	T	2	0	0
638,679	A	G	2	0	0
638,681	A	–	6	0	0
638,687	–	T	92	0	0
638,779	–	AT	885	26	63.25
639,664	C	A	210	6	61.52
639,874	A	G	60	2	71.77
639,934	A	T			
Total			5,825	98	36.22
cM					0.21

1. Prepare the first round of PCR reactions (a–f) using F_1 pollen genomic DNA equivalent to approximately 20 or 30 crossover molecules per reaction (estimated from Subheading 3.6. **Steps 1–34**), as below.

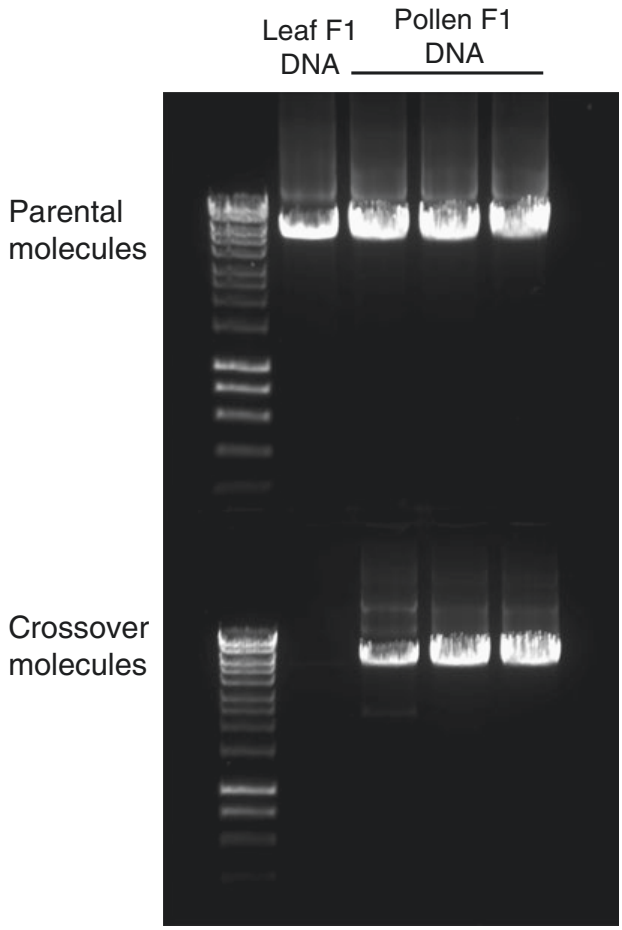

Fig. 5 Crossover and parental molecule amplification from leaf and pollen genomic DNA following qPCR analysis. A representative 1 % agarose ethidium bromide stained gel showing nested crossover and parental PCR products. Here the products of the first round of allele-specific PCR performed during qPCR analysis are used as templates for a second round of allele-specific PCR (Subheading 3.8). Crossover molecules are specifically amplified from pollen and not leaf F_1 genomic DNA

Components	Volume (μl)
F_1 pollen genomic DNA (20 or 30 crossovers) or F_1 leaf genomic DNA	–
10× Ex Taq buffer	2
dNTP, each 2.5 mM	2
1st ASO F (5 μM)	2
1st ASO R for COs or ASO/UO R for parentals (5 μM)	2
Ex Taq (5 U/μl)	0.1
Nuclease-free water	–
Total	20

(a) F_1 leaf DNA, ASO Col F/Col R or UR for parental.

(b) F_1 pollen DNA, ASO Col F/Col R or UR for parental.

(c) Mutant F_1 pollen DNA, ASO Col F/Col R or UR for parental.

(d) F_1 leaf DNA, ASO Col F/Ler R for crossover.

(e) F_1 pollen DNA, ASO Col F/Ler R for crossover.

(f) Mutant F_1 pollen DNA, ASO Col F/Ler R for crossover.

2. Run the first round of PCR as for single molecule amplifications (Subheading 3.6. **step 9**), but with a reduced number of PCR cycles (e.g., 16–25) (*see* **Note 5**).
3. Dilute the first PCR product 20-fold for crossover molecules and 1000–2000-fold for parental molecules, using 10 mM Tris–HCl (pH 8).
4. Use the diluted PCR products as templates for qPCR reactions with a pair of universal forward and reverse primers (UO F, UO R) (Table 5).

Components	Volume (μl)
Diluted first PCR products	2
10× SYBR green	2
10× DNA Taq buffer	2
dNTP, each 2.5 mM	2
UO F (5 μM)	2
UO R (5 μM)	2
Go Taq (5 U/μl)	0.2
Nuclease-free water	7.8
Total	20

Table 5
Sequences of universal oligonucleotides used for qPCR analysis

Hotspot	Orientation	Sequence (5'–3')
3a	Forward	GTTCAAGCTTAAAGGGAAATCG
3a	Reverse	GCCCATGACTCGGTGTAAAT
3b	Forward	GACGAGCTCGAAGGAATCAG
3b	Reverse	ATGCTGAGGCCTATCATTGC
RAC1/RPP9	Forward	GCCGATTTGATGCCTCACTT
RAC1/RPP9	Reverse	AAGGAATCCAGGATCGCTGT

5. Run real time thermal cycler analysis using the following parameters:

   1 cycle of,

   95 °C for 30 s;

   27 cycles of,

   95 °C for 30 s,

   60 °C for 20 s,

   72 °C for 30 s (detection);

   1 cycle of,

   72 °C for 1 min;

   Hold at 4 °C.

6. The diluted products of the first round of allele-specific PCR from **step 3** can also be used as templates for a second round of allele-specific PCR (as in Subheading 3.6. **steps 11–14**), in order to test specificity of crossover amplifications from pollen and leaf genomic DNA (Fig. 5).

7. Calculate the relative crossover amplification enrichment from F_1 pollen genomic DNA (sample) compared to F_1 leaf DNA (control) by using the $2^{-\Delta\Delta Ct}$ method, as below [45]. The relative fold change between sample and control is calculated as:

   Relative fold change = 2^−((*C* − *P*) − (CL − PL)).

   C: Ct from crossover amplification from F_1 pollen DNA.

   P: Ct of parental amplification from F_1 pollen DNA.

   CL: Mean Ct of crossover amplification from F_1 leaf DNA.

   PL: Mean Ct of parental amplification from F_1 leaf DNA.

Here, *C*t (the threshold cycle) reflects the PCR cycle number at which fluorescence from accumulating amplicon products within a reaction crosses the threshold above background signal [45]. Therefore, in Table 6 the fold change of wild type crossovers compared between pollen and leaf genomic DNA is 2^−((14.62 − 27.69) − (22.78 − 27.49)) = 328.56.

3.9 High Throughput Crossover Sequencing Library Construction

In order to sample greater numbers of crossovers than is feasible from Sanger sequencing of single molecule amplification products, we have developed a method to mass amplify crossovers and generate high-throughput sequencing libraries (Fig. 1). Here, multiple independent PCR amplifications are performed, each containing an estimated 1–3 crossover template molecules, based on previous titrations experiments. Low numbers of estimated crossovers are used per reaction in order to evenly amplify from a large number of independent molecules.

Table 6
Crossover and parental molecule amplification from leaf and pollen genomic DNA measured using qPCR

ASO combination	Template	Code	Ct	Mean Ct	Fold change	Fold change mean	Fold change St. Dev.
Parentals	F_1 leaf	a	27.4	27.49			
	F_1 leaf		27.16				
	F_1 leaf		27.9				
	F_1 pollen wild type	b	27.69				
	F_1 pollen wild type		27.69				
	F_1 pollen wild type		27.67				
	F_1 pollen *arp6*	c	27.65				
	F_1 pollen *arp6*		27.66				
	F_1 pollen *arp6*		27.61				
Crossovers	F_1 leaf	d	23.03	22.78	0.79	1.04	0.36
	F_1 leaf		22.65		0.87		
	F_1 leaf		22.65		1.45		
	F_1 pollen wild type	e	14.62		328.56	363.65	52.99
	F_1 pollen wild type		14.58		337.79		
	F_1 pollen wild type		14.23		424.61		
	F_1 pollen *arp6*	f	15.53		170.07	195.86	23.27
	F_1 pollen *arp6*		15.29		202.25		
	F_1 pollen *arp6*		15.15		215.27		

Relative crossover enrichment from allele-specific amplication of leaf and pollen Col/Ler F_1genomic DNA, comparing between wild type and *arp6* mutants using the $2^{-\Delta\Delta Ct}$ method (Subheading 3.8, **steps 1–7**). a–f indicate the template and primer combination used, as described in Subheading 3.8, **step 1**. Three technical qPCR replicates were performed using first PCR products from reactions a-f as templates. These data are one of the biological replicates published in [12]

1. Prepare eight 96-well PCR plates for the first round of allele-specific PCR, as below. Based on the previous titration of crossover molecules (Subheading 3.6, **steps 1–35**), add the amount of pollen genomic DNA to each well, such that each contains an estimated 1–3 crossovers (e.g., 1/concentration of crossovers × −ln(0.35)).

Components	Volume (μl)	100 reactions
F_1 pollen genomic DNA (1–3 crossovers)	–	–
10× Ex Taq buffer	1	100
dNTP, each 2.5 mM	1	100
1st ASO F (5 μM)	1	100
1st ASO R (5 μM)	1	100
Ex Taq (5 U/μl)	0.05	5
Nuclease-free water	–	–
Total	10	Each 10

2. Run the first round of allele-specific PCR as for single crossover molecule amplifications (Subheading 3.6, **step 9**).
3. Dilute the PCR products 20-fold by adding 190 μl of 10 mM Tris–HCl (pH 8) to each well.
4. Prepare a reaction cocktail for eight 96-well PCR plates for the second round of allele-specific PCR, as below.

Components	Volume (μl)	100 Reactions
Diluted first PCR product	1	Each
10× Ex Taq buffer	1	100
dNTP, each 2.5 mM	1	100
2nd ASO F (5 μM)	1	100
2nd ASO R (5 μM)	1	100
Ex Taq (5 U/μl)	0.05	5
Nuclease-free water	4.95	495
Total	10	Each 9

5. Aliquot 9 μl to each well using an automatic pipette.
6. Add 1 μl of diluted 1st round PCR amplifications to each well of the 2nd round PCR plate, using an eight channel pipette.
7. Seal the plate and mix by centrifuging at 500 × *g* for 1 min.
8. Run the second round of PCR, as for single molecule crossover amplifications (Subheading 3.6, **step 14**).
9. Add 2.5 μl of 5× DNA loading buffer to 12 wells of each 96-well plate and analyze 5 μl of the PCR product using a 1.2% agarose gel containing ethidium bromide, to confirm the presence of amplification products.

10. Pool the second round PCR products from each 96-well plate (900 µl) in a 2 ml tube.
11. Add 0.1 volume of 3 M sodium acetate and 1 vol of isopropanol.
12. Incubate at −20 °C for 2 h.
13. Centrifuge the samples at 16,000 × *g* for 30 min at 4 °C.
14. Remove the supernatant, wash the pellet with 1 ml of 70% EtOH, centrifuge again for 5 min at 4 °C.
15. Remove the supernatant and dry the pellet at room temperature for 5 min.
16. Dissolve the pellets in 100 µl of TE and store at −80 °C.
17. Analyze the PCR products using gel electrophoresis with a 1.2% agarose and ethidium bromide staining.
18. Excise the amplification product band of expected size from the gel.
19. Purify the PCR products using a gel extraction kit.
20. Measure purified DNA amount using a fluorometer and dilute to 20 ng/µl.
21. Sonicate 2 µg of DNA in 100 µl of TE in a 1.5 ml tube to 300–500 bp by using a Bioruptor (high setting, 30 s ON–30 s OFF for 15 min). The size of DNA fragmentation can be varied according to distribution of polymorphisms. For example, if polymorphisms are relatively sparse in the analyzed region then using longer DNA fragments may be beneficial.
22. Analyze the PCR products by gel electrophoresis using a 2% agarose gel containing ethidium bromide.
23. Excise the 300–400 bp region from the gel containing the sonicated crossover PCR products.
24. Purify the PCR products using a gel extraction kit.
25. Measure the purified DNA amount using a fluorometer.
26. Generate a DNA sequencing library, according to the manufacturer's instructions.
27. Sequence the library using paired-end reads of 150 bp length, for example, using an Illumina miSeq or NextSeq500 instrument.

3.10 Analysis of Pollen Typing High Throughput Crossover Sequencing Data

1. It is first necessary to perform Sanger sequencing of the two parental haplotypes analyzed during pollen typing (e.g., Col-0 and Ler-0). Use these data to generate two FASTA files containing the sequence of the two parents within the amplified region. A suggested data analysis pipeline is described below.
2. Build a bowtie index for both sequences, or similar file for alternative alignment methods [46].

3. Trim adapter sequences from reads. Suitable programs include FastX or Trimmomatic (http://hannonlab.cshl.edu/fastx_toolkit/index.html) [47].
4. Paired-end reads should be separated into forward read and reverse read FASTQ files.
5. Align each read set to the Col-0 and Ler-0 amplicon sequences from **steps 1** and **2**, allowing only uniquely matching alignments. Suitable programs include BWA, bowtie or bowtie2 [46, 48].
6. This will result in four alignment BAM or SAM files (end1.col, end1.ler, end2.col, end2.ler), which should be sorted and indexed using samtools [49].
7. Use Rsamtools to input alignment data into R [50].
8. Filter for reads pairs, i.e., those with matching read names, where one read uniquely aligns to the Col-0 sequence and one uniquely aligns to the Ler-0 sequence. The read genotypes and alignment coordinates should match the ASO configuration used during allele-specific PCR amplification (Fig. 1).
9. Use the filtered read pairs for analysis, normalized by the total number of mapped, filtered reads. Crossover values can also be assigned to all intervening base pairs between the innermost polymorphisms present in paired crossover read alignments, weighted by interval sizes.

4 Notes

1. The quality of DNA obtained is strongly dependent on the vortexing step. Vortexing for longer periods increases the number of disrupted pollen grains, which increases DNA yield. However, vortexing also increases shearing of DNA. This is a problem due to the long length of pollen typing amplicons (5–10 kb). The vortexing step should be optimized for local laboratory equipment and conditions in order to maximize crossover amplification from pollen genomic DNA.
2. The final concentration of pollen genomic DNA is less important than the quantity of amplifiable crossover molecules. As crossover and parental amplicons are large, for example ~10 kb, it is important that DNA molecules remain as intact as possible. This is evident by a clear high molecular weight band when DNA is analyzed by agarose gel electrophoresis and ethidium bromide staining (Fig. 2). The number of amplifiable molecules is increased by careful manipulation of pollen genomic DNA. For example, never vortex or vigorously mix the DNA and resuspend pellets gently by hand.

3. Frequently ASOs show template specificity only at higher temperatures (e.g., >60.5) and not lower temperatures (e.g., <59.5) during gradient PCR optimisation (e.g., ASO_6 in Fig. 3). In this case ensure to use higher annealing temperatures during all amplifications.
4. PCR conditions, including cycle number, should be carefully optimized, as described in **steps 1–16** of Subheading 3.6, using pollen and leaf F_1 genomic DNA. Conditions should be identified where no amplification is observed from leaf F_1 DNA templates, after for example, 35 PCR cycles. Therefore, when amplifying crossover molecules from pollen F_1 DNA under the same conditions, any observed amplification is classified as positive and an absence of amplification is classified as negative.
5. It is important to use a minimal number of PCR cycles (e.g., 16–25), otherwise saturation of amplified PCR products can mask differences between samples. Varying first PCR cycle numbers should be tested until an approximately a 30–500-fold enrichment of crossovers is detected from F_1 pollen DNA, compared to F_1 leaf DNA (Subheading 3.8, **step 7** and Table 6).

References

1. Osman K, Higgins JD, Sanchez-Moran E et al (2011) Pathways to meiotic recombination in Arabidopsis thaliana. New Phytol 190:523–544
2. Kauppi L, Jeffreys AJ, Keeney S (2004) Where the crossovers are: recombination distributions in mammals. Nat Rev Genet 5:413–424
3. Villeneuve AM, Hillers KJ (2001) Whence meiosis? Cell 106:647–650
4. Barton NH, Charlesworth B (1998) Why sex and recombination? Science 281:1986–1990
5. Keeney S, Neale MJ (2006) Initiation of meiotic recombination by formation of DNA double-strand breaks: mechanism and regulation. Biochem Soc Trans 34:523–525
6. De Massy B (2013) Initiation of meiotic recombination: how and where? Conservation and specificities among eukaryotes. Annu Rev Genet 47:563–599
7. Mercier R, Mézard C, Jenczewski E et al (2014) The molecular biology of meiosis in plants. Annu Rev Plant Biol. doi:10.1146/annurev-arplant-050213-035923
8. Baudat F, Imai Y, de Massy B (2013) Meiotic recombination in mammals: localization and regulation. Nat Rev Genet 14:794–806
9. Choi K, Henderson IR (2015) Meiotic recombination hotspots – a comparative view. Plant J 83:52–61
10. Coop G, Przeworski M (2006) An evolutionary view of human recombination. Nat Rev Genet 8:23–34
11. Yelina NE, Choi K, Chelysheva L et al (2012) Epigenetic remodeling of meiotic crossover frequency in Arabidopsis thaliana DNA methyltransferase mutants. PLoS Genet 8, e1002844
12. Choi K, Zhao X, Kelly KA et al (2013) Arabidopsis meiotic crossover hot spots overlap with H2A.Z nucleosomes at gene promoters. Nat Genet 45:1327–1336
13. Drouaud J, Khademian H, Giraut L et al (2013) Contrasted patterns of crossover and non-crossover at Arabidopsis thaliana meiotic recombination hotspots. PLoS Genet 9:e1003922
14. Tiemann-Boege I, Calabrese P, Cochran DM et al (2006) High-resolution recombination patterns in a region of human chromosome 21 measured by sperm typing. PLoS Genet 2:e70
15. Baudat F, de Massy B (2007) Cis- and trans-acting elements regulate the mouse Psmb9 meiotic recombination hotspot. PLoS Genet 3:e100
16. Cole F, Keeney S, Jasin M (2010) Comprehensive, fine-scale dissection of homologous recombination outcomes at a hot spot in mouse meiosis. Mol Cell 39:700–710

17. Berg IL, Neumann R, Sarbajna S et al (2011) Variants of the protein PRDM9 differentially regulate a set of human meiotic recombination hotspots highly active in African populations. Proc Natl Acad Sci U S A 108:12378–12383
18. Kauppi L, May CA, Jeffreys AJ (2009) Analysis of meiotic recombination products from human sperm. Methods Mol Biol. doi:10.1007/978-1-59745-527-5
19. Jeffreys AJ, Kauppi L, Neumann R (2001) Intensely punctate meiotic recombination in the class II region of the major histocompatibility complex. Nat Genet 29:217–222
20. Arbeithuber B, Betancourt AJ, Ebner T, Tiemann-Boege I (2015) Crossovers are associated with mutation and biased gene conversion at recombination hotspots. Proc Natl Acad Sci U S A 112:2109–2114
21. De Boer E, Jasin M, Keeney S (2015) Local and sex-specific biases in crossover vs. noncrossover outcomes at meiotic recombination hot spots in mice. Genes Dev 29:1721–1733
22. De Boer E, Jasin M, Keeney S (2013) Analysis of recombinants in female mouse meiosis. Methods Mol Biol 957:19–45
23. Drouaud J, Mézard C (2011) Characterization of meiotic crossovers in pollen from Arabidopsis thaliana. Methods Mol Biol 745:223–249
24. Yelina NE, Lambing C, Hardcastle TJ et al (2015) DNA methylation epigenetically silences crossover hot spots and controls chromosomal domains of meiotic recombination in Arabidopsis. Genes Dev 29:2183–2202
25. Khademian H, Giraut L, Drouaud J, Mézard C (2013) Characterization of meiotic noncrossover molecules from Arabidopsis thaliana pollen. Methods Mol Biol 990:177–190
26. Auton A, McVean G (2012) Estimating recombination rates from genetic variation in humans. Methods Mol Biol 856:217–237
27. Charlesworth B, Charlesworth D (2010) Elements of evolutionary genetics. Roberts and Company, Englewood, CO
28. Weigel D, Nordborg M (2015) Population genomics for understanding adaptation in wild plant species. Annu Rev Genet 49:315–338
29. Auton A, McVean G (2007) Recombination rate estimation in the presence of hotspots. Genome Res 17:1219–1227
30. Fearnhead P (2006) SequenceLDhot: detecting recombination hotspots. Bioinformatics 22:3061–3066
31. Drouaud J, Mercier R, Chelysheva L et al (2007) Sex-specific crossover distributions and variations in interference level along Arabidopsis thaliana chromosome 4. PLoS Genet 3:12
32. Giraut L, Falque M, Drouaud J et al (2011) Genome-wide crossover distribution in Arabidopsis thaliana meiosis reveals sex-specific patterns along chromosomes. PLoS Genet 7:e1002354
33. Salomé PA, Bomblies K, Fitz J et al (2012) The recombination landscape in Arabidopsis thaliana F2 populations. Heredity (Edinb) 108:447–455
34. Wijnker E, Velikkakam James G, Ding J et al (2013) The genomic landscape of meiotic crossovers and gene conversions in Arabidopsis thaliana. Elife 2, e01426
35. Shilo S, Melamed-Bessudo C, Dorone Y et al (2015) DNA crossover motifs associated with epigenetic modifications delineate open chromatin regions in Arabidopsis. Plant Cell 27:tpc.15.00391. doi:10.1105/tpc.15.00391
36. Hellsten U, Wright KM, Jenkins J et al (2013) Fine-scale variation in meiotic recombination in Mimulus inferred from population shotgun sequencing. Proc Natl Acad Sci U S A 110:19478–19482
37. Dooner HK (1986) Genetic fine structure of the BRONZE Locus in maize. Genetics 113:1021–1036
38. Brown J, Sundaresan V (1991) A recombination hotspot in the maize A1 intragenic region. Theor Appl Genet 81:185–188
39. Saintenac C, Faure S, Remay A et al (2011) Variation in crossover rates across a 3-Mb contig of bread wheat (Triticum aestivum) reveals the presence of a meiotic recombination hotspot. Chromosoma 120:185–198
40. Chodavarapu RK, Feng S, Bernatavichute YV et al (2010) Relationship between nucleosome positioning and DNA methylation. Nature 466:388–392
41. Zilberman D, Coleman-Derr D, Ballinger T, Henikoff S (2008) Histone H2A.Z and DNA methylation are mutually antagonistic chromatin marks. Nature 456:125–129
42. Zhang X, Bernatavichute YV, Cokus S et al (2009) Genome-wide analysis of mono-, di- and trimethylation of histone H3 lysine 4 in Arabidopsis thaliana. Genome Biol 10:R62
43. Liu S, Yeh C-T, Ji T et al (2009) Mu transposon insertion sites and meiotic recombination events co-localize with epigenetic marks for open chromatin across the maize genome. PLoS Genet 5:13
44. Copenhaver GP, Nickel K, Kuromori T et al (1999) Genetic definition and sequence analysis of Arabidopsis centromeres. Science 286:2468–2474

45. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–408
46. Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. Nat Methods 9:357–359
47. Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30:2114–2120
48. Li H, Durbin R (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25:1754–1760
49. Li H, Handsaker B, Wysoker A et al (2009) The Sequence Alignment/Map format and SAMtools. Bioinformatics 25:2078–2079
50. R Development Core Team (2012) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna