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TECHNICAL ADVANCE

A new seed-based assay for meiotic recombination in *Arabidopsis thaliana*

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Summary

Meiotic recombination is a fundamental biological process that plays a central role in the evolution and breeding of plants. We have developed a new seed-based assay for meiotic recombination in Arabidopsis. The assay is based on the transformation of green and red fluorescent markers expressed under a seed-specific promoter. A total of 74 T-DNA markers were isolated, sequenced and mapped both physically and genetically. Lines containing red and green markers that map 1–20 cM apart were crossed to produce tester lines with the two markers linked in *cis* yielding seeds that fluoresced both in red and green. We show that these lines can be used for efficient scoring of recombinant types (red only or green only fluorescing seeds) in a seed population derived from a test cross (backcross) or self-pollination. Two tester lines that were characterized during several generations of backcross and self-pollination, one in the background of ecotype Landsberg and one in the ecotype Columbia, are described. We discuss the number of plants and seeds to be scored in order to obtain reliable and reproducible crossing over rate values. This assay offers a relatively high-throughput method, with the benefit of seed markers (similar to the maize classical genetic markers) combined with the advantages of Arabidopsis. It advances the prospect to better understand the factors that affect the rate of meiotic crossover in plants and to stimulate this process for more efficient breeding and mapping.

Keywords: meiosis, recombination assay, genetic markers, genome mapping.

Introduction

Meiotic recombination is a fundamental aspect of sexual reproduction and evolution, and provides the physical basis for understanding genetics. This process is also of profound interest for plant breeding because it generates new genetic variation upon which selection can be applied. In the vast majority of species, meiotic recombination is essential for proper chromosome segregation as well as for generating diversity within a species. It is an exquisite process because of its complexity and its precision, involving a combination of structural aspects of chromosome behavior and enzymatic processes for DNA recombination. Much of the recent progress in the understanding of meiotic recombination, comes from cytogenetics, genetics and molecular analyses carried out in *Saccharomyces cerevisiae* (see reviews by Bishop and Zickler, 2004; Zickler and Kleckner, 1999). Genes and candidate genes have been identified that affect pairing of homologs, induction of DNA double strand breaks (DSBs), 3′ single strand DNA (ssDNA) tail resection and strand invasion as well as formation, maintenance and resolution of Holliday junctions.

In plants, the behavior of chromosomes during meiosis has been studied for many years for a number of reasons: several species have large chromosomes that have been historically exploited for cytological analysis, anthers are a convenient source for the analysis of cells undergoing meiosis and recombination-related mutants have been relatively easy to identify owing to their full or partial sterility (see review in Dawe, 1998). The molecular basis of many of these mutants and their effect on recombination

is still obscure. The Arabidopsis revolution has stimulated forward and reverse genetics approaches to study meiotic recombination in plants, owing to the full genomic sequence (Arabidopsis Genome Initiative, 2000) and to the availability of mapped T-DNA insertion lines (http://signal.salk.edu). Several plant genes have been identified that are homologous to the yeast recombination genes as described in a recent review (Schuermann *et al.*, 2005). We mention below some of the genes that are most relevant to meiotic recombination. The recombination process is initiated by a DNA DSBs catalyzed by *SPO11*, homologs of which were found in Arabidopsis (Hartung and Puchta, 2000). A mutant in one of these genes, *AtSPO11*, shows a severe reduction in meiotic recombination (Grelon *et al.*, 2001). The AtSpo11-mediated breaks are not repaired in an Arabidopsis *mre11* mutant (Puizina *et al.*, 2004), nor in a *rad50* mutant (Bleuyard *et al.*, 2004). In both mutants, chromosomes do not synapse and plants are sterile. This supports the role of Rad50 and Mre11 as part of the complex that senses DNA breaks and triggers the recruiting of the DNA repair machinery, thus affecting meiotic recombination as mentioned above, as well as mitotic repair and telomere elongation (Bundock and Hooykaas, 2002; Gallego and White, 2001; Puizina *et al.*, 2004). Homologs of Rad51, a protein that promotes invasion of the broken DNA into a homologous sequence, have also been found in Arabidospsis (Bleuyard and White, 2004; Doutriaux *et al.*, 1998). A mutant in one of these genes, *AtXRCC3*, is not affected in synapsis, but shows chromosome fragmentation in the later stages of meiosis, after pachytene, and is sterile (Bleuyard and White, 2004). Msh4 is thought, together with Msh5, to recognize Holliday junctions and clamp homologs (Snowden *et al.*, 2004). A mutant in an Arabidopsis homolog, *AtMSH4*, has been recently identified (Higgins *et al.*, 2004). It shows partial and delayed synapsis as well as partial sterility. Other genes, such as the Arabidopsis *Solo Dancer*, a cell-cycle-related gene (Azumi *et al.*, 2002) or genes that regulate the pairing of homologs, *AHP2* in Arabidopsis (Schommer *et al.*, 2003), and *PHS1* in maize (Pawlowski *et al.*, 2004), have been isolated. The effect of most of the above genes on the rates of meiotic crossing over has not been determined, either because of the high sterility of the mutants or because it has not been tested. Conversely, X-ray-sensitive (*xrs*) mutants with altered rates of meiotic recombination have been isolated in Arabidopsis but the underlying genes are not known (Masson and Paszkowski, 1997).

The Arabidopsis model system has not yet been fully exploited to study the genetic and environmental factors that affect meiotic crossing over, in part because it is difficult to monitor recombination rates in this species. The density of DNA markers is very high in Arabidopsis (Peters *et al.*, 2003), however, it remains time consuming to grow plants, extract DNA and genotype the plants. A recombination assay has been recently developed in Arabidopsis, whereby recombination is determined based on the ratio of seedlings that are resistant to both kanamycin and hygromycin (Barth *et al.*, 2000). While this assay is a significant advance that facilitates monitoring of recombination events it has still several limitations addressed in the Discussion. Morphological markers are also useful, however, the number of seedling markers is limited in Arabidopsis and other markers can be monitored only at later stages of development, e.g. flowering mutations. In maize, geneticists have made a remarkable use of seed markers to monitor recombination. A classical example, still used in the study of meiotic recombination, is the linkage group on chromosome 9 involving *C1*, *Shrunken1*, *Bronze1* and *Waxy1* (Fu *et al.*, 2002; McClintock, 1951).

Taking inspiration from the work on maize, and from the recent report of seed-expressed fluorescent proteins (Stuitje *et al.*, 2003) we have initiated the construction of new tester lines to facilitate the monitoring of meiotic recombination in Arabidopsis. These lines contain a pair of genetic markers, linked in *cis*, namely GFP and RFP that can be scored in seeds, thus combining the simplicity of seed markers with the advantages of the Arabidopsis model system. We describe here the production and molecular genetic analysis of such lines in the Arabidopsis ecotypes, Columbia and Landsberg.

Results

Mapping of GFP and RFP seed-specific markers

The expression of GFP or RFP under a seed-specific promoter (napA) can be visualized using different filters (see Experimental procedures), thus enabling to distinguish between the expression of each marker separately, e.g. only green or only red seeds, or seeds that are both red and green (see Figure 1). These features make GFP and RFP ideally suited to be used as genetic markers to study crossing over between linked loci or for rapid gene mapping. We have mapped insertion sites of GFP and RFP seed-markers in two Arabidopsis ecotypes, namely Columbia and Landsberg. Transformation of the GFP and RFP markers with the pFLUAR100 and pFLUAR101 constructs (Stuitje *et al.*, 2003) was followed by isolation of T-DNA insertion sites using the TAIL-PCR method (Liu *et al.*, 1995). Only 17% of transformants were amplified and sequenced, the rest could not be mapped by this method. The number of plants used for TAIL-PCR, together with the number of mapped insertion sites is described in Table 1. Altogether, 74 sites were mapped in the two ecotypes, 43 in Columbia and 31 in Landsberg and 25 GFP versus 49 RFP markers.

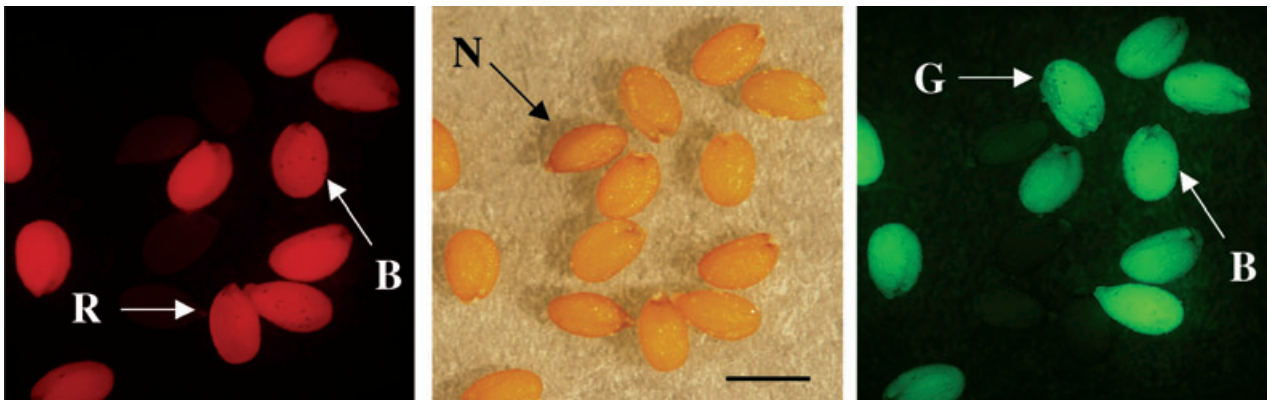

Figure 1. GFP and RFP fluorescent seeds.
Visualization of Arabidopsis seeds under visible light (middle panel) or using UV light and two different filters, one for RFP (left panel) and one for GFP (right panel). R: only RFP is expressed, G: only GFP is expressed, B: both RFP and GFP are expressed and N: none of the fluorescence markers is expressed. The size of the black horizontal line in the central panel is 0.5 mm.

Table 1 Number of transformants and mapped insertion sites for the seed-expressed GFP and RFP markers

Ecotype	Marker	No. plants	Mapped[a]
Columbia	GFP	124	12
	RFP	203	31
Landsberg	GFP	40	13
	RFP	124	18

[a]Number of T-DNA insertions sites that were isolated and mapped by TAIL-PCR.

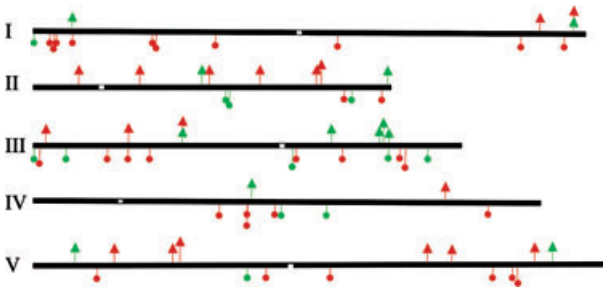

Figure 2. Schematic representation of the distribution of the fluorescence markers along the five chromosomes of *Arabidopsis thaliana*.
The mapping positions of TAIL-PCR products are represented in green for the GFP and in red for the RFP markers. The white box shows the centromeres. The relative location of mapped markers is indicated by triangles for ecotype Landsberg erecta and as circles for ecotype Columbia.

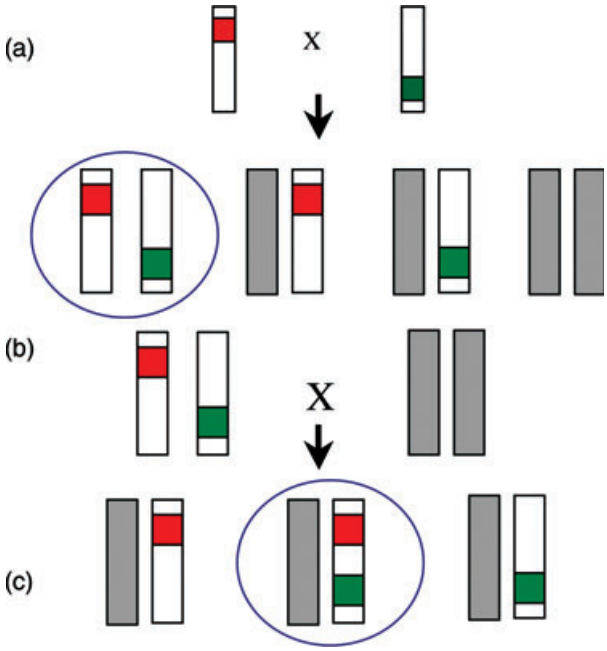

Figure 3. Construction of meiotic tester lines.
Pairs of plants containing the GFP and RFP markers located on homologous chromosomes and mapping 0–20 cM apart were crossed (a). Seeds expressing both GFP (green box) and RFP (red box) were selected, grown and backcrossed with WT plants (gray chromosomes) (b). Progeny seeds of such backcross expressing both GFP and RFP result from a crossover event between the two markers (c).

The distribution of the markers along the chromosomes is shown in Figure 2. Each marker could be associated with a precise physical position on the chromosome and to a genetic locus, based on the Lister and Dean recombinant inbred genetic map (Lister and Dean, 1993) that has been superimposed on the physical map (The Arabidopsis Information Resource: http://www.arabidopsis.org).

The making of meiotic tester lines with GFP and RFP markers linked in cis

The process we used to construct lines that contain a pair of mapped seed-specific GFP and RFP markers linked in *cis* is described in Figure 3. As a first step, a pair of transformants

Table 2 Genetic segregation and mapping of the GFP and RFP seed markers in tester lines

Tester	Markers in pairs[a]	Fluorescent seeds	Non-fluorescent seeds	$P(\chi^2)$[b]	Chr.	Location (cM)	Insertion site[c] (bp)
Col3-4/20	GCo39	470	176	0.188	3	4	256516
	RCo59	267	91	0.855	3	20	5361637
Col3-54/59	GCo31	189	50	0.152	3	54	14211755
	RCo131	202	69	0.861	3	59	15101840
Le5-11/22	GLe70	177	82	0.0136	5	11	2053974
	RLe64	407	120	0.237	5	21	4184075
Le3-42/42	GLe51	168	46	0.236	3	42	10065734
	RLe88	152	74	0.012	3	42	9801389

Chr.: Chromosome.
[a]GCo: GFP in Columbia; RCo: RFP in Columbia; GLe: GFP in Landsberg erecta; RLe: RFP in Landsberg erecta.
[b]Calculated by deviation from the expected 3:1 segregation.
[c]Chromosome coordinates at the insertion site of the left T-DNA border.

Table 3 Recombination rates between GFP and RFP markers as determined in the second and third back-cross generations (BC2 and BC3) between Le5-11/22 and wt

	Seed phenotype					
Generation	Red	Green	Both	None	Total	% Rec.
BC2						
wt Le × Le5-11/22 1 BC1	20	13	112	125	270	
wt Le × Le5-11/22 1 BC1	9	9	80	107	205	
Total	29	22	192	232	475	10.7
BC3						
wt Le × Le5-11/22 3 BC2	1	3	27	37	68	
wt Le × Le5-11/22 4-2 BC2	4	2	62	54	122	
wt Le × Le5-11/22 4-3 BC2	4	12	105	110	231	
wt Le × Le5-11/22 5 BC2	5	8	91	95	199	
wt Le × Le5-11/22 9-1 BC2	3	1	50	65	119	
wt Le × Le5-11/22 11 BC2	4	6	88	105	203	
wt Le × Le5-11/22 13 BC2	1	4	48	66	119	
wt Le × Le5-11/22 16-1 BC2	3	3	64	68	138	
wt Le × Le5-11/22 16-2 BC2	2	5	50	60	117	
wt Le × Le5-11/22 17-1 BC2	9	4	68	57	138	
Total	36	48	653	717	1454	5.8

was chosen as parental lines for crossing. Two lines were selected, one containing a GFP and the other an RFP marker that are located on the same chromosome, within a genetic distance of 0–20 cM, based on the Lister and Dean (1993) genetic map (Figure 3a). In the T_2 generation of each independent plant, the segregation ratio of the marker was measured and the lines that passed the chi-squared test [$P(\chi^2) > 0.01$] for a 3:1 Mendelian segregation were used for crossing (Figure 3a and Table 2). Two lines, GLe70 and RLe88, barely passed the 3:1 test, nevertheless, they were maintained in subsequent analysis because when large numbers of seeds were analyzed in backcrosses (Table 3), or via self-pollination (Table 4 and data not shown), they showed a very accurate 1:1 or 3:1 segregation, respectively. From the segregation data obtained for these two lines (Table 2), it could be interpreted that they follow a 2:1 segregation. It is unlikely for two reasons: first, if this was the

Table 4 Recombination rates between GFP and RFP markers as determined in F_2 self-pollinated seeds derived from plants heterozygous for the two markers in tester Le5-11/22

	Red	Green	Both	None	Total	% Rec.
Le5-11/22 11-1 BC2 -F2	15	15	460	126	616	
Le5-11/22 11-2 BC2-F2	9	13	361	120	503	
Le5-11/22 12 BC2 -F2	7	8	227	80	322	
Le5-11/22 13 BC2 -F2	19	21	498	149	687	
Le5-11/22 15 BC2 -F2	13	21	359	117	510	
Le5-11/22 16-1 BC2 -F2	8	5	136	39	188	
Le5-11/22 16-2 BC2 -F2	23	18	450	148	639	
Le5-11/22 17-1 BC2 -F2	10	14	288	77	389	
Le5-11/22 18 BC2 -F2	18	11	433	151	613	
Le5-11/22 9-1 BC2 -F2	10	10	324	90	434	
Le5-11/22 3-1 BC2 -F2	3	2	71	24	100	
Le5-11/22 4-2 BC2 -F2	16	15	415	111	557	
Total	151	153	4022	1232	5558	5.5

case they would not have the 1:1 or 3:1 segregation in large seed populations as mentioned above; secondly, because a 2:1 ratio would suggest that seeds homozygous for the dominant locus are not viable. This was not the case as homozygous lines could be produced for these loci.

Seeds resulting from crosses between the parental lines containing the linked GFP and RFP markers were screened by microscope and seeds that fluoresce in both red and green were selected (Figure 3a). These seeds contain the GFP and RFP markers, each on a different homolog (circled pair in Figure 3a). Such seeds were grown and the resulting plants were crossed with the isogenic corresponding wild-type plants, namely Landsberg or Columbia (Figure 3b). In such test cross, seeds that express both markers are derived from a crossover between homologous chromosomes, because the wild type did not contribute any marker. In these seeds, the two markers are linked in *cis* (Figure 3c).

In Table 2, we describe four tester lines, two in ecotype Columbia and two in ecotype Landsberg.

Two testers, line Le5-11/22, and line Col3-4/20 containing GFP and RFP markers 11 and 16 cM apart, respectively, (according to the Lister and Dean maps) are described below. The assortment of the markers in tester line Le5-11/22 was followed during three generations of self and backcross. First, it was crossed again with wild-type plants (BC2 generation). Two independent plants were analyzed and a total of 475 seeds were counted. The recombination rate obtained, based on the number of recombinant seeds (29 red and 22 green only seeds), is 10.7% (Table 3). In order to increase the sample size, and follow fluctuations in recombination rates, a third backcross with a bigger population was made (Table 3). From a total of 1454 seeds derived from 12 plants, we obtained a recombination rate of approximately 5.8%. A major difference between BC2 and BC3 is that BC2 plants were grown in a greenhouse where temperatures were relatively hot during flowering time, while BC3 and all subsequent experiments were carried out in a growth chamber with a controlled environment (16 h of day light, 19°C day and night). We have preliminary results suggesting that heat might account for such differences. In addition, the seeds resulting from self-pollination of all these 12 plants were also collected and analyzed (Table 4). The fluctuation in the recombination rate among self-pollinated independent plants was less strong than in the backcross (probably because of the larger number of seeds counted), and the mean rate of recombination was similar to that found in the backcross (5.5%). In order to identify the potential source of variation in the experiment, the same seeds from the self-pollination of the plants heterozygous for the markers that gave the 5.5% value were counted again in an independent manner by a different person and the rate of recombination was 5.3% showing that the 'counter' effect has only a minor effect and thus that the scoring is relatively straightforward.

The rates of meiotic crossover were also studied in the background of ecotype Columbia, in tester line Col3-4/20. This tester was backcrossed for four generations to WT Columbia (BC1-4) and crossover rates were determined in self-progeny (F2) of these plants. Results are shown in Table 5. The crossover rate was very stable for four generations ranging between 15.39 and 16.48%. Moreover, it was similar to the 16 cM expected from the Lister and Dean maps.

For both tester lines described above, we found that each marker segregated in a typical Mendelian manner, giving the expected 3:1 ratios. For tester Le5-11/22, the segregation of each marker in F2 was as follows: the ratio of red versus non-red fluorescent seeds was $(151 + 4022)$: $(1232 + 153) = 3.01$; the ratio of green versus non-green fluorescent seeds was $(153 + 4022)$:$(1232 + 151) = 3.02$ (Table 4). For tester Col3-4/20, the segregation of each marker in F2 was as follows: the ratio of red versus non-red fluorescent seeds was $(560 + 4729)$: $(1254 + 490) = 3.03$; the ratio of green versus non-green fluorescent seeds was $(490 + 4729)$: $(1254 + 560) = 2.88$ (Table 5).

Discussion

We have established a new meiotic recombination assay in Arabidopsis whereby the segregation of genetic markers linked in *cis*, namely GFP and RFP, can be scored in seeds. Seventy-four markers were mapped. These GFP and RFP markers can be combined in pairs linked in *cis* to form recombination tester lines that potentially enable monitoring of meiotic crossover rates throughout the Arabidopsis genome. Barth *et al.* (2000) showed that transgenic markers conferring resistance to kanamycin or hygromycin can be used to monitor recombination via identification of Arabidopsis seedlings resistant to both antibiotics. The seed assay described here has several advantages compared with the double antibiotic resistance assay discussed below. Recombination is determined at the seed, rather than at the seedling stage, saving time and effort of growing plants in tissue culture. Moreover, with the double antibiotic assay, the two markers are linked in *trans* (each on a different homolog) and it takes one cross between the testers plus a backcross to identify the recombinant individuals. The second cross is work-intensive as it requires a large number of hybridizations. Whereas when markers are linked in *cis*, recombination rates can be determined in the selfed F2 population, thus requiring only one round of crossing. Finally, and more importantly, determining the effect of a recessive mutation on recombination is much more complex with markers in *trans* than in *cis*.

In this study, four tester lines were made, two of which were monitored for several generations in test crosses as well as in self-pollinated F2 populations. One tester, Col3-4/20, in the Columbia background, showed consistent results

Table 5 Recombination rates between GFP and RFP markers as determined in self-pollinated F2 seeds derived from plants heterozygous for the two markers in tester Col3-4/20

	Seed phenotype					
Generation	Red	Green	Both	None	Total	% Rec.
BC1-F2						
wt ColXCol3-4/20 BC1-F2-1	34	33	307	64	438	
wt ColXCol3-4/20 BC1-F2-3	43	53	413	131	640	
Total	77	86	720	195	1078	16.48
BC2-F2						
wt ColXCol3-4/20 BC2-F2-1	74	56	533	121	784	
wt ColXCol3-4/20 BC2-F2-2	44	41	386	115	586	
wt ColXCol3-4/20 BC2-F2-3	67	32	420	124	643	
wt ColXCol3-4/20 BC2-F2-4	45	43	420	134	642	
Total	230	172	1759	494	2655	16.40
BC3-F2						
wt ColXCol3-4/20 BC3-F2-2	26	35	257	58	376	
wt ColXCol3-4/20 BC3-F2-3	21	16	206	59	302	
wt ColXCol3-4/20 BC3-F2-5	31	22	264	68	385	
Total	78	73	727	185	1063	15.39
BC4-F2						
wt ColXCol3-4/20 BC4-F2-3	25	42	278	67	412	
wt ColXCol3-4/20 BC4-F2-4	40	37	316	85	478	
wt ColXCol3-4/20 BC4-F2-5	35	29	305	80	449	
wt ColXCol3-4/20 BC4-F2-7	44	24	320	73	461	
wt ColXCol3-4/20 BC4-F2-8	31	27	304	75	437	
Total	175	159	1523	380	2237	16.25
Grand total	560	490	4729	1254	7033	16.25

for four generations showing the expected 3:1 ratio for each marker and the recombination values between the markers were as expected from the Lister and Dean genetic map [(Lister and Dean, 1993) TAIR http://www.arabidopsis.org], i.e. approximately 16 cM (Table 5).

The other tester analyzed in detail was Le5-11/22 in the Landsberg background. This tester also gave the expected 3:1 ratio as shown in Table 4. This ratio was also observed in additional experiments performed in the laboratory aimed at determining genetic and environmental factors that affect meiotic recombination (data not shown). The distance between the markers, approximately 5.5 cM, was also highly reproducible, in test crosses (Table 3) as well as in selfed progeny in various generations (Table 4) and also in several additional experiments (data not shown). Surprisingly, this distance is about half that predicted based on the Lister and Dean genetic map. We have investigated the possible causes for such discrepancy. Both the Lister and Dean results and our results are based on thorough mapping, in different experiments and environments. Therefore it is unlikely that the observed discrepancy is caused by some statistical variation. In order to double-check our results, we designed a new primer pair specific for the left border of the T-DNA and for the genomic sequence as predicted by the TAIL-PCR results (see Experimental procedures). The PCR fragments obtained from both GFP and RFP positions (data not shown) had the expected size and were sequenced, confirming the original TAIL-PCR results, i.e. the predicted approximately 11 cM distance between markers. Therefore erroneous mapping of the T-DNA insertion is not the cause for this distance discrepancy. We further tested whether some odd combination of multicopy insert together with silencing could give rise to the observed data. *A priori*, this seems unlikely as we found very stable distance measurements and precise 3:1 segregation ratio for the markers. Nevertheless the possibility of silencing was tested by extracting DNA from four pools, each containing 25 seedlings, derived from non-fluorescent seeds. DNA was analyzed by PCR using GFP or RFP-specific primers. No PCR product could be amplified from the non-fluorescent seeds whereas products were obtained from red or green fluorescent seeds. A positive control, namely actin, was amplified from both the fluorescent and non-fluorescent seeds. Because this work was performed using pools instead of single plants we checked the possibility of false-negatives that might have been caused by a dilution effect. One fluorescent seed was mixed with 49 non-fluorescent seeds, and under such conditions we could amplify a GFP or RFP product. This suggests that we did not monitor false-negative seeds and that there is no significant silencing of the transgenes in the experiment. A Southern blot analysis was performed for the two markers and complex hybridization patterns were observed for both markers suggesting the presence of multicopy inserts. The stable and precise 3:1 segregation of each marker, the reproducible distance between the two markers and the lack of false-negative seeds suggests that we deal with a single locus rather than a single copy T-DNA insertion. Although this is not an ideal situation as it may be prone to silencing,

we did not observe silencing in our experiments and moreover, if such silencing were to occur in future generations, it would be easy to detect through alterations in the segregation ratio of the markers that should be checked in each experiment. We cannot rule out the presence of T-DNA-induced rearrangements in the region between the markers that would affect recombination rates. In support of this possibility is our failure to obtain TAIL-PCR products from the right border or inverse PCR products. It is also possible that one of the T-DNA insertions in Le5-11/22 has caused some alterations in chromatin structure that affects recombination rates. Future studies should clarify the causes for the distance discrepancy in Le5-11/22; in the meantime, this tester gives highly reproducible results for genetic distances as well as for markers segregation and can be used for the study of recombination.

Reproducibility in recombination rates is achieved only if enough seeds are analyzed. It is likely that even within a given experiment, there are fluctuations between individual plants as a result of differences in the microenvironment where the plant grew. Further studies should analyze environmental effects. In order to minimize such effects, we recommend counting seeds from several plants. We observed that in backcrosses, scoring 100 or more seeds from at least six plants gives reproducible results. For the seeds derived from self-pollinated plants, a higher number of seeds should be analyzed, e.g. 400 or more per plant. An advantage of seed markers over DNA markers or whole plant markers is that one can score manually thousands of seeds in a few hours without the need for expensive tools and without the need of a large plant population. Moreover, the system can be automated through methods such as scanning of seeds laid on a surface with a scanner equipped with the appropriate filters for fluorescence detection, or through flow cytometry such as COPAS^{TM} automated sorting systems (Union Biometrica Inc., Somerville, MA, USA).

Seed testers can be used to study DNA recombination in different genetic and environmental backgrounds. Mutants, which may contain selective markers, such as resistance to kanamycin or Basta, can be tested using the GFP/RFP seed testers because they do not have any antibiotic as selective marker. Testing the effect of dominant mutants can be carried out by crossing to the tester and determining their effect on recombination already in the F2 of this cross. Recessive mutants must be introgressed in the tester background to test their effect on recombination. This requires an additional generation (via selfing or backcross to the mutant) compared with dominant mutants. Environmental effects can be determined by treating the heterozygous tester. Another benefit of having mapped tester lines with easy-to-score markers is to facilitate mapping and map-based cloning in Arabidopsis.

In summary we have developed a high-throughput assay for meiotic crossing over that opens the prospect to genetically dissect meiotic recombination via the analysis of several genes through over-expression or mutagenesis. It might also be used for efficient mapping of mutations and isolation of the underlying genes. The system can be improved by automation and by adding other seed markers (such as YFP and CFP) to the system. Three or more linked seed markers would enable to extend the utility of the system to the study of crossover interference.

Experimental procedures

Plant transformation

The binary plasmids pFLUAR100 and pFLUAR101 containing the GFP and RFP markers, respectively, under the regulation of the specific Napin promoter (for description see Stuitje *et al.*, 2003) were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation (Shen and Forde, 1989). Transformation of Arabidopsis (ecotypes Landsberg erecta and Columbia) was carried out by the floral dip method as described (Clough and Bent, 1998). Separate *Agrobacterium* cultures were grown overnight to stationary phase in LB medium at 28°C. In single T-DNA transformation experiments, the cells were concentrated to an OD_{600} of approximately 1.8 in 5% sucrose and 0.5 MS salts. Silwett L-77 was added to a final concentration of 0.02% prior to dipping. Transformed seeds were selected by fluorescence detection with an Olympus (Melville, NY, USA) SZX12 stereomicroscope with a reflected light fluorescence unit URF-LT and filter sets SZX-MG for GFP and SZX-MGFPA for RFP. The frequency of transformation, as determined by fluorescence microscopy, was close to 2%.

DNA isolation

Genomic DNA was extracted from the T-DNA insertion lines by grinding two to three leaves in the presence of liquid nitrogen, using a disposable grinder driven by an electric drill. A volume of 0.7 ml of pre-heated isolation buffer (0.8 M NaCl, 0.12 M Tris–HCl pH 7.5, 25 mM EDTA, 0.8% CTAB and 0.8% sarkosyl) was added to each tube. An equal volume (0.7 ml) of chloroform/octanol (24:1) was added to each tube, mixed and centrifuged at ca. 20 000 ***g*** for 5 min in a microfuge. This step was repeated twice. Two volumes of storage buffer (75% ethanol, 0.2 M sodium acetate) were added to the supernatant and DNA was recovered by centrifugation at 20 000 ***g*** for 20 min. Finally, the pellet was resuspended in 30 μl water.

TAIL-PCR procedure

The insertion sites of the GFP and RFP markers in the transformed plants were mapped using thermal asymmetric interlaced (TAIL)-PCR (Liu *et al.*, 1995). The specific primers LB1: 5′-GTTCACG-TAGTGGGCCATCG-3′, LB2: 5′-ATCGCCCTGATAGACGGTTTT-3′, and LB3: 5′-CGTGGACCGCTTGCTGC-3′ were designed from the T-DNA left border with melting temperatures of 61, 57 and 57°C, respectively, as recommended (Liu *et al.*, 1995). The degenerated primers used were identical to those described (Liu *et al.*, 1995).

Unpurified TAIL-PCR products from the third round of amplification were directly sequenced (DNA Sequencing Unit, The Weizmann Institute of Science) using LB3 as primer. The sequence of the T-DNA border was removed and the rest of the sequence was used for sequence analysis using BLAST against 'The Arabidopsis Information Resource' (TAIR http://www.arabidopsis.org) database. Using this approach both physical and genetic map locations of the insertions were determined.

Mapping the insertions in Le5-11/22 and Col3-4/20 by PCR

The mapped insertions were re-checked by PCR using primer LB3 (described above) specific for the left border of the T-DNA and primers for the genomic sequence as follows: GLe70 (green insertion in Le5-11/22), 5′-AAATTCTCGCCTTGGTTCTG-3′; RLe64 (red insertion in Le5-11/22), 5′-TTACTTCTTTCCTCCACTAC-3′; GCo39 (green insertion in Col3-4/20), 5′-GGGGAAAGGGCTGAGAAG-3′; RCo59 (red insertion in Col3-4/20), 5′- ACCATAGTCTTTGCGTTTAG-3′. The annealing temperature of all of the PCR reactions was 55°C

Calculation of crossing over rates in self-pollinated seeds of an heterozygous tester

Red only or green only seeds (resulting from crossover) contain either both gametes resulting from recombination, or one gamete of the wt parent (no fluorescent marker) and one recombinant gamete (see data in bold in Table 6).

Gametes of parental types have a frequency of $(1-r)/2$, where (r) is the recombination rate and the frequency of recombinant gametes is $r/2$. The frequency of green only and red only seeds (c) equals the sum of all the frequencies indicated in bold in Table 6. The recombination rate can therefore be calculated from the following quadratic equation:

$$4[(r - r^2)/4] + 2(r^2/4) = c$$

that is simplified to:

$$r^2 - 2r + 2c = 0.$$

and is solved using the quadratic formula:

$$r = \frac{-b - \sqrt{b^2 - 4ac}}{2a}$$

where $a = 1$, $b = -2$ and $c =$ [(number of green + red only seeds)/ total number of seeds].

Table 6 Genotypes of seeds derived from self-pollination of plants heterozygote for both the GFP and RFP markers

Gametes (frequency)	RG [(1−*r*)/2]	rg [(1−*r*)/2]	Rg (*r*/2)	rG (*r*/2)
RG [(1−*r*)/2]	RRGG	RrGg	RRGg	RrGG
rg [(1−*r*)/2]	RrGg	rrgg	**Rrgg [$(r-r^2)/4$]**	**rrGg [$(r-r^2)/4$]**
Rg (*r*/2)	RRGg	**Rrgg [$(r-r^2)/4$]**	**RRgg [$(r^2/4)$]**	RrGg
rG (*r*/2)	RrGG	**rrGg [$(r-r^2)/4$]**	RrGg	**rrGG [$(r^2/4)$]**

Gametes are indicated as R or G for the RFP and GFP seed markers, respectively. Lower case r or g corresponds to the wild-type colorless gamete.

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