Pollen tetrad-based visual assay for meiotic recombination in *Arabidopsis*

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Recombination, in the form of cross-overs (COs) and gene conversion (GC), is a highly conserved feature of meiosis from fungi to mammals. Recombination helps ensure chromosome segregation and promotes allelic diversity. Lesions in the recombination machinery are often catastrophic for meiosis, resulting in sterility. We have developed a visual assay capable of detecting Cos and GCs and measuring CO interference in *Arabidopsis thaliana*. This flexible assay utilizes transgene constructs encoding pollen-expressed fluorescent proteins of three different colors in the *qrt1* mutant background. By observing the segregation of the fluorescent alleles in 92,489 pollen tetrads, we demonstrate (*i*) a correlation between developmental position and CO frequency, (*ii*) a temperature dependence for CO frequency, (*iii*) the ability to detect meiotic GC events, and (*iv*) the ability to rapidly assess CO interference.

cross-over | meiosis | tetrad | gene conversion | interference

Meiosis, the reductive division of the genome in preparation for fertilization, is a critical phase in the life cycle of sexually reproducing organisms. During meiosis, homologous chromosomes interact, resulting in the heritable rearrangement of DNA through reciprocal exchange between homologous chromosomes [cross-over (CO)] or gene conversion (GC). COs serve to stabilize homolog pairing during meiosis and also provide an adaptive mechanism for generating favorable allelic combinations (1, 2). Conversely, because COs are generated by DNA double-strand breaks, they represent a significant liability to the cell if they are not repaired efficiently and with high fidelity. The cell balances these costs and benefits by tightly regulating meiotic recombination, including the imposition of CO interference in most organisms. There is surprising diversity in the execution and regulation of meiotic recombination in different organisms, ranging from utilization of different protein components to divergence in the use of entire regulatory pathways, such as interference mechanisms (3, 4). Because of this diversity, researchers use a variety of model systems to study meiosis, including fungi, plants, insects, nematodes, and mammals.

Despite the need to establish a broad-based understanding of meiotic recombination, the preponderance of the current mechanistic data comes from a single organism, *Saccharomyces cerevisiae*. Budding yeast has a number of advantages for the study of meiotic processes. It has a convenient collection of easily assayable markers (auxotrophic alleles, etc.), which makes it reasonable to obtain statistically relevant data sets. Furthermore, the haploid life cycle of yeast makes it possible to detect recombination events directly in the products of meiosis (more accurately, their clonal propagants) without having to do subsequent crosses. Perhaps most importantly, tetrad analysis is available in yeast (5). Each meiosis in yeast produces four spores that remain packaged together, making it possible to track and measure all of the products of any recombination or segregation event. For example, tetrad analysis can detect the non-Mendelian 3:1 segregation of markers characteristic of GC unambiguously (6, 7). Tetrad analysis also allows both products of a reciprocal event (e.g., CO) to be observed, providing informational redundancy that reduces the chances of misscoring.

Using the advantages offered by yeast as an exemplar, we have developed an assay system for detecting meiotic recombination events in *Arabidopsis thaliana*. The system combines the *qrt* mutant background, which yields tetrads of meiotically related pollen grains, with a series of transgenic marker genes that encode pollen-expressed fluorescent proteins excitable by different wavelengths of light (8, 9). Using this system, we are able to visualize CO and GC events directly and reliably in gametes in a way that enables tetrad analysis. Because the system utilizes three different fluorescent proteins, it also facilitates the measurement of CO interference. A different seed-based assay has been described by Melamed-Bessudo *et al.* (10). The seed-based assay also has the advantage of being high throughput and using fluorescent proteins as visual reporters, but it lacks the ability to assay recombination directly in the gametes, and it does not incorporate the advantages of tetrad analysis. Our pollen tetrad-based visual assay lends itself to the rapid assessment of COs, GC, and interference in virtually any mutant background capable of producing at least some pollen. Here we use this system to show that the developmental position of flowers producing gametes influences CO frequency; we do so by demonstrating that tetrads produced from secondary and tertiary axes have a higher recombination frequency (RF) in comparison to primary bolts. We also show that CO frequency correlates with temperature. Last, we demonstrate that the assay system can be used to detect meiotic GC and measure CO interference in *Arabidopsis*.

Results and Discussion

Genetic intervals that can be visually assayed for recombination were created by transforming *qrt* plants with marker genes encoding red, yellow, or cyan fluorescent proteins (CFPs) under control of the LAT52 promoter (Fig. 1*A*) (11, 12). Transformed seeds were selected by using either kanamycin or glufosinate to yield 2,752 T_1 plants. Each of these lines was screened by using a number of criteria to ensure their usefulness for subsequent meiotic analysis. First, pollen tetrads from each T_1 plant were observed under the fluorescence microscope. Each T-DNA insertion event in the T_1 will result in a hemizygous locus; therefore, if we observed consistent 2:2 segregation of the fluorescent signal in the pollen tetrads, we assumed a single-locus insertion (Fig. 2*B*). This assumption has two caveats.

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Abbreviations: CO, cross-over; GC, gene conversion; LMS-PCR, ligation-mediated suppression-PCR; FTL, fluorescent-tagged line; I1, interval 1; I3, interval 3; I5, interval 5; CFP, cyan fluorescent protein; ECFP, enhanced CFP; YFP, yellow fluorescent protein; EYFP, enhanced YFP; RF, recombination frequency; NPD, nonparental ditype; DCO, double cross-over.

See Commentary on page 3673.

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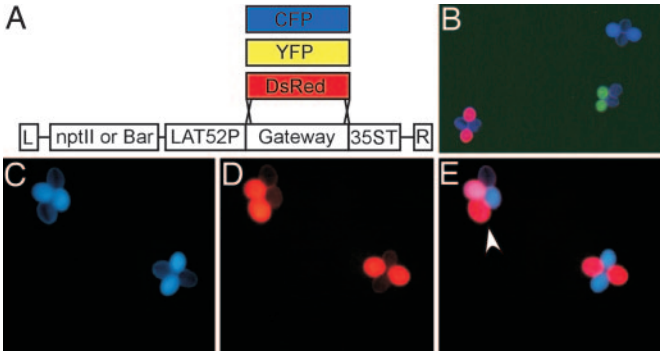

Fig. 1. Fluorescent markers in pollen tetrads. (*A* and *B*) An *Agrobacterium* T-DNA construct (*A*) containing ECFP, EYFP, or DsRed driven by the LAT52 promoter and a selectable marker conferring resistance to either kanamycin (*npt* II) or glufosonate (Bar) was used to transform *qrt1 Arabidopsis* seed resulting in T_1 plants expressing the fluorescent protein in their pollen (*B* shows pollen from three different plants). (*C–E*) Crossing lines with differently colored transgenes on the same chromosome (*C* and *D*) enables detection of CO events in the interval between the transgenes (*E*, merge of *C* and *D* with arrow indicating recombinant tetrad).

Multiple tandem copies of the marker gene at a single locus will also segregate in a consistent 2:2 pattern, and silenced or disrupted copies will not be detected. Lines that showed more complicated patterns, including 4:0, 3:1, 1:3, and 0:4, were assumed to be the result of multiple insertions, partial silencing, or disruption of the fluorescent marker genes and were not given further consideration. We also rejected lines that showed very weak expression of the fluorescent protein.

Genomic DNA flanking the T-DNA in single-insert lines with strong expression was obtained by using ligation-mediated suppression (LMS)-PCR and subsequently sequenced to map each insertion precisely (Fig. 2) (13). Because multiple LMS-PCR fragments can be indicative of multiple or complex integrations,

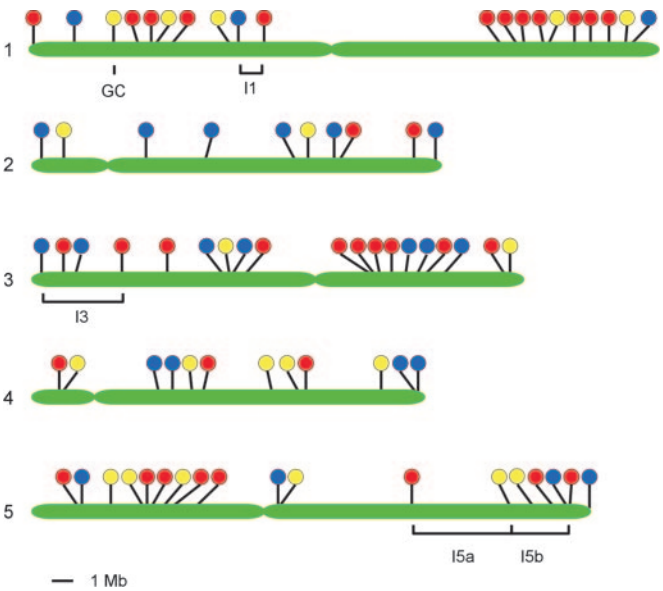

Fig. 2. Map of fluorescent transgenes. The chromosomal (green bars) insertion site of the transgene carried by each FTL lines is indicated by a red (DsRED), yellow (EYFP), or cyan (ECFP) circle. The genetic intervals (I1, I3, I5a, and I5b) used in this study are delineated with brackets, and the locus used to detect GC event (GC) is also marked. The *Arabidopsis* Information Resource ''chromosome map tool'' was used to place T-DNA insertion points on the physical map (15).

lines resulting in complex LMS-PCR patterns were discarded. This sequencing also allowed us to differentiate insertions in annotated genes from those in intergenic regions. When using these markers to measure recombination frequencies (as described below), it is important that disruption of genes by the marker does not confound the results, particularly when using the markers in mutant backgrounds where synthetic effects would be difficult to predict. Accordingly, we proceeded with only those lines that gave strong 2:2 expression patterns with markers inserted into intergenic regions. These were designated fluorescent-tagged lines (FTLs).

Lines selected for further use were also subjected to T_2 segregation analysis. T_1 plants were allowed to self, and their T_2 progeny were visually scored to ensure that plants with 4:0, 2:2, or 0:4 segregation of the transgene in their pollen tetrads were occurring in the expected 1:2:1 Mendelian ratio. This final check serves three purposes: (*i*) it confirms the previous assumption of a single-locus insertion, (*ii*) it reveals any phenotypes resulting from carrying the marker in the homozygous state, and (*iii*) it confirms the stability of the marker in an additional generation. Of the original 2,752 positive transformants, 79 lines passed our quality control criteria [Fig. 2; supporting information (SI) Table 1].

To detect COs, pairs of lines carrying differently colored markers on the same chromosome were crossed to produce F_1 progeny carrying both markers in trans and creating a genetic interval bounded by two visible markers. COs can be observed in the pollen tetrads produced by this F_1 plant by visually scoring the segregation of the two fluorescent proteins. Without a CO in the interval, the tetrad will have two grains of one color and two grains of the other color. A CO in the interval will result in a tetrad with one bicolored grain, one noncolored grain, and two monocolored grains (Fig. 1 *C* and *D*). It should also be noted that two markers in a cis configuration would also be useful for assaying COs but would result in a different pattern for recombinant and nonrecombinant classes. Several hundred tetrads can be easily scored in 1 hr by using this assay, thereby facilitating the collection of very large data sets.

As specific examples, we crossed two FTL lines carrying insertions on chromosome 1, FTL992 CFP), and FTL1313 (DsRed) to create ''interval 1'' (I1) and two insertions on chromosome 3, FTL1500 (CFP), and FTL1371 (DsRed) to create ''interval 3'' (I3; Fig. 2). Assuming an average genomic RF of 200 kb/cM (119,186 kb/597 cM), these intervals are expected to be 6.4 and 19.1 cM, respectively (www.arabidopsis.org). We scored 9,181 and 8,919 tetrads from F_1 plants carrying markers that define I1 and I3, respectively, and we observed a map distance of 6.2 cM for I1 and 23.1 cM for I3. Thus, the markers themselves do not appear to inhibit local recombination. I1 and I3 are interstitial regions that do not encompass any special chromosomal domains such as centromeres or rDNA arrays (14). The collection of FTL lines that we have established provides the basis for establishing test intervals of a variety of sizes on any of the *Arabidopsis* chromosomes.

Using methods described by Papazian (15), it is possible to calculate the expected number of nonparental ditype (NPD) tetrads resulting from double COs in a single interval using the frequency of single COs, which are observed as tetratypes (15). Applying this method to our data set, we would expect to observe 19 and 304 NPDs for I1 and I3, respectively; we observed only 2 and 51. This paucity of NPDs is consistent with our observation of CO interference using dual-interval analysis, described below.

Arabidopsis produces multiple flowers, each of which is an independent source of gametes. The production of flowers in a temporal series and in a variety of developmental positions serves as a potential source of natural variability in CO frequencies. To assess this potential, we measured CO frequencies in I1 and I3 (25,249 and 23,830 total tetrads, respectively), using flowers from primary bolts (1°), branches on primary bolts (2°),

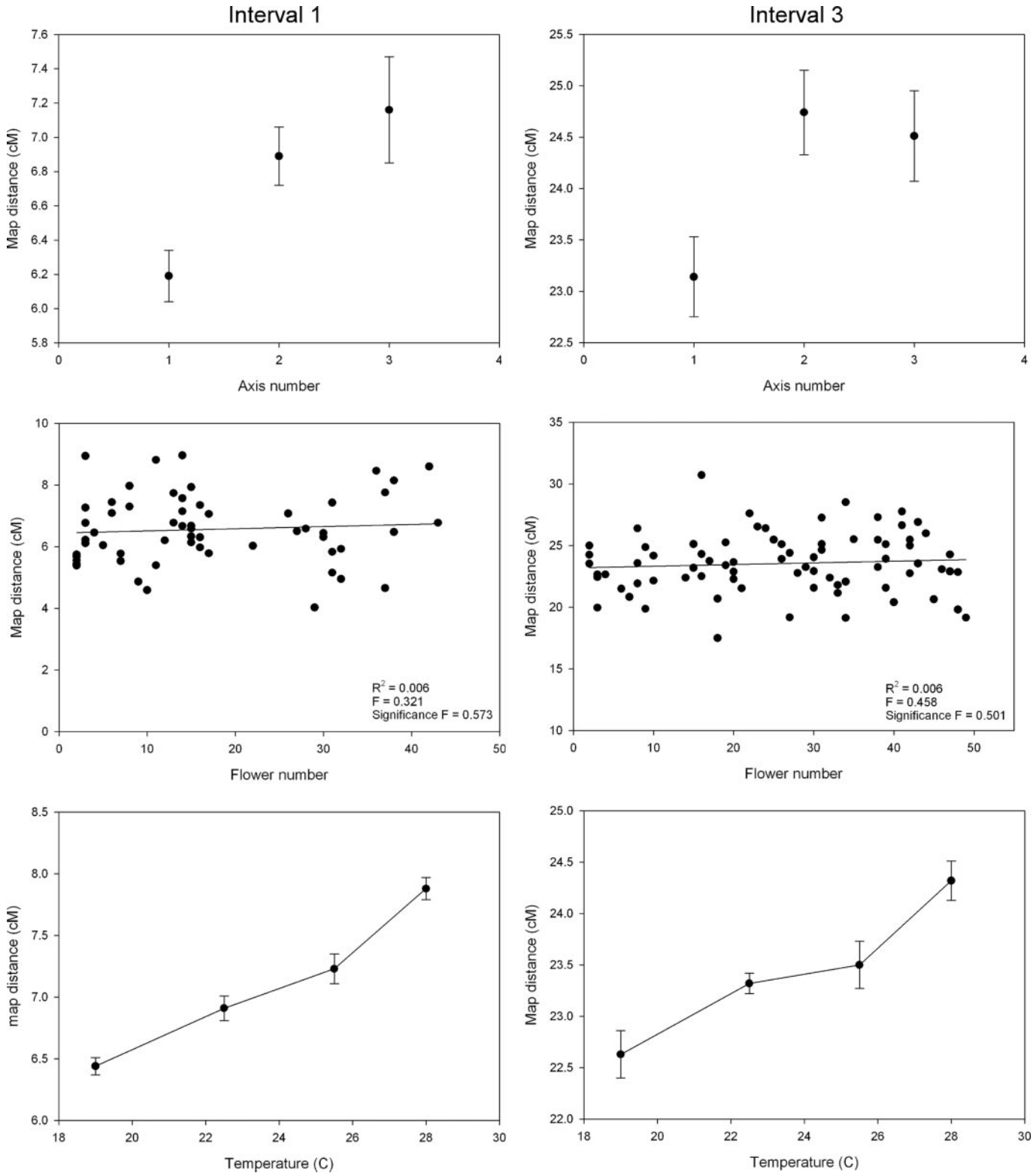

Fig. 3. Genetic analyses using the visual assay system. (*Top*) Map distances in centimeters were measured by using flowers from the primary bolt (1), branches of the primary bolt (2), and branches of branches (3) in both I1 (*Left*) and I3 (*Right*). (*Middle*) As a control for flower age, map distances were also measured in individual flowers, beginning with the first and ending with the last, produced by the primary axis in I1 (*Left*) and I3 (*Right*). (*Bottom*) The influence of temperature during flowering was measured in I1 (*Left*) and I3 (*Right*) by using flowers from the primary axis. Error bars (*Top* and *Bottom*) are based on standard error.

and branches of branches (3°). Surprisingly, we observed that both 2° and 3° flowers produced tetrads with significantly higher CO frequencies compared with 1° flowers (Fig. 3 *Top*). This result may indicate a regulatory connection between developmental programs and CO control in *Arabidopsis*. It is possible that this difference may result from the fact that 1° flowers are

derived from the apical meristem, whereas 2° and 3° flowers are derived from lateral meristems. It is also possible that, on average, 2° and 3° flowers are produced when the plant itself is older (an age effect). To explore this possibility, we measured CO frequencies in I1 and I3, using individual flowers along the length of each axis. For each flower, we scored at least 100 tetrads. Although there was variation in map distances from flower to flower, the regression from first to last flower revealed no correlation between flower age and map distances on any of the axes (Fig. 3 *Middle*). Thus, we conclude that the difference in CO frequencies we observed in 1° or 2° and 3° flowers is due to developmental position rather than age. Because plants have multiple independent germ lines that arise in different developmental contexts, they provide a unique opportunity to examine the relationship between the regulation of meiotic recombination and development.

Plants, being sessile, often experience dramatic ranges of temperatures in the wild. To measure the sensitivity of CO frequency to temperature, we grew plants in a 20°C growth chamber under long days until they began to bolt, and then we shifted them to 19°C, 22.5°C, 25.5°C, or 28°C under constant light. We then assayed RFs in I1 and I3 (10,988 and 20,739 total tetrads, respectively). In both intervals, we observed significant increases in map distances across the temperature range (Fig. 3 *Bottom*). These data are consistent with previous observations in both plants and animals. In *Hordeum vulgare*, *Vicia faba*, and *Drosophila melanogaster*, there is a general trend toward higher levels of COs with higher temperature (16–18). This trend may represent a regulatory connection between temperature sensing and CO control, or the mechanical process of CO may be sensitive to temperature. Studies in *Locusta migratoria* and *Allium ursinum* indicate that extreme temperatures can interfere with chromosome mechanics such as synapsis, leading to a decrease in CO frequency (19, 20). Interestingly, temperature studies in *Neurospora crassa* reveal a temperature optima for COs (in one interval) at moderate temperatures (21).The fluorescent assay system described here will enable more detailed genetic analysis of the relationship between environmental conditions and meiotic recombination. Whatever their causes, the results of the temperature and the developmental experiments indicate that care must be taken in collecting CO data from *Arabidopsis*. Growth conditions should be well controlled, and data should be collected from equivalent developmental tissues.

To demonstrate that the visual assay system can be used to measure CO interference, we crossed two FTL lines carrying insertions on chromosome 5, FTL1273 (DsRed), and FTL1659 [yellow fluorescent protein (YFP)], to create F_1 progeny carrying both markers in trans. Self-seeds from these F_1 plants were planted, and pollen from the F_2 plants were visually scored to identify recombinant individuals with the two markers in cis. Recombinant F_2 individuals were then crossed to a line carrying a third FTL insertion on chromosome 5, FTL993 (CFP), to produce progeny carrying all three markers (Fig. 4). These three markers define two adjacent genetic intervals, interval 5a (I5a) and I5b (Fig. 2). By following the segregation pattern of all three markers, it is possible to distinguish: (*i*) meioses lacking COs, (*ii*) meioses with single COs in either interval, and (*iii*) all classes (two, three-, and four-stand) of double cross-overs (DCOs, Fig. 4). We scored 1,247 tetrads from these plants and observed RFs of 16 in I5a and 24 in I5b. Multiplying the product of these frequencies by the total number of gametes scored ($1{,}247 \times 4 = 4{,}988$), we would predict 190 DCO gametes in the combined interval; however, we observed only 60. These data yield an interference value [i.e., 1 − (observed DCO/expected DCO)] of 0.68. In the past, measuring interference in *Arabidopsis* was difficult, because the large data sets necessary to accumulate enough DCOs for analysis were time-consuming to generate. With our visual assay, interference measurements can routinely be obtained, thereby enhancing the value of *Arabidopsis* as a model system for meiotic processes.

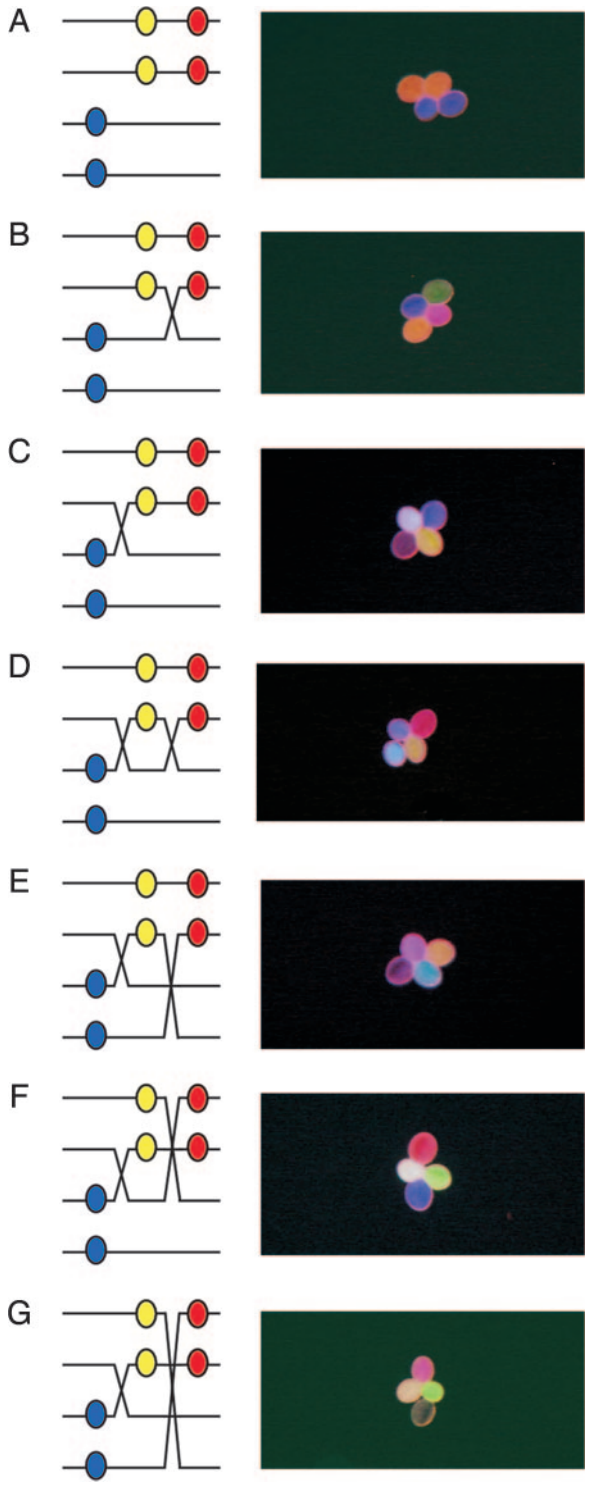

Fig. 4. Measuring interference. DsRed (red circle), EYFP (yellow circle), and ECFP transgene (cyan circle) define two adjacent genetic intervals (I5a and I5b; see text) on chromosome 5. The four chromatids present after replication can experience no COs (*A*), single COs in either interval (*B* and *C*), and DCOs in the combined interval including two-strand DCOs (*D*), both kinds of three-strand DCOs (*E* and *F*), and four-strand DCOs (*G*) in the two intervals. Each of these events can be distinguished by observing the segregation of the transgenes.

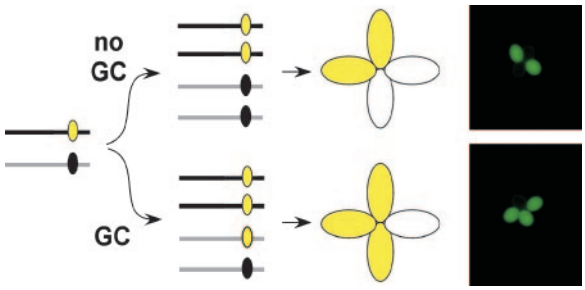

Fig. 5. Detecting GC. A heterozygous plant with one fluorescent EYFP allele (yellow circle) and one mutant nonfluorescent eyfp allele (black circle) can be used to detect meiotic GC events in pollen tetrads.

One of the unique advantages of tetrad-based recombination analysis is the ability to identify GC events unambiguously by their classic non-Mendelian 3:1 segregation pattern. We have created a modified version of the FTL system that makes it possible to detect 3:1 segregation patterns. A plant carrying a nonfluorescent allele of FTL567 (yfp) was generated by using ethyl methanesulfonate mutagenesis (Fig. 2). Sequencing this allele revealed a G to A transition at nucleotide 95 in the YFP gene, causing a Gly-32 to Asp mutation that resulted in the loss of fluorescence. This yfp/yfp line was crossed to the original parental FTL567 line to generate F_1 plants that were heterozygous at the fluorescent transgenic locus (YFP/yfp). Pollen tetrads that do not experience GC at this locus will have two fluorescent and two nonfluorescent grains (Fig. 5). A GC event spanning the locus will generate three fluorescent and one nonfluorescent grains (or vice versa). After scoring 4,033 tetrads, we observed six tetrads with a 3:1 segregation ratio. We ignored 1:3 tetrads because of the possibility that occasional nonviable pollen grains might yield false positives. Assuming that the directionality of GC is not biased toward either allele, we conclude that, at this locus, GC occurred in 1/336 meioses. To control for false 3:1 positives due to reversion of the yfp allele to a fluorescent state, we scored 8,783 yfp/yfp tetrads and never observed a fluorescent grain [$P < 0.0001$ by two-tailed Fisher's exact test (22)]. This is an unambiguous observation of meiotic GC in *Arabidopsis* by using tetrad analysis. Additional test loci will need to be developed to generate genome-wide estimates of GC frequencies. In contrast to this test locus, which we specifically designed to detect GC, we did not observe any cases of 3:1 segregation in any of the hemizygous lines used to generate the I1 and I3 data described above.

The FTL assay system described here enables rapid and inexpensive detection of COs across the *A. thaliana* genome, measurement of CO interference, and quantification of GC frequencies. These tools will facilitate the analysis of recombination phenotypes in mutant lines. It is particularly advantageous that these analyses can be done in essentially isogenic lines. Moreover, because large numbers of tetrads can be scored easily, experiments that would have been difficult in the past because of laborious crossing schemes can now be done with relative ease. In demonstrating the use of the assay system, we showed that temperature is correlated with CO frequency in *Arabidopsis*. More surprisingly, we discovered a correlation between the developmental position of flowers producing gametes and CO frequency. This finding raises interesting questions about why a connection between developmental position and meiotic recombination should exist, and how it might be mediated. This quick and practical assay system will now enable experiments designed to probe how environmental cues like temperature and developmental signals influence meiotic recombination at a mechanistic level.

Materials and Methods

Plant Materials. *A. thaliana qrt1-1* in the Landsberg-0 background (CS8845) and *qrt1-2* in the Columbia-3 background (CS8846) were used to generate the FTLs in this study. Seeds were sown on Pro-mix (Premier Horticulture, Quakertown PA) and stratified for 3–4 days at 4°C. Plants were germinated and grown under long-day conditions (18 h light) at 20°C unless otherwise noted. Temperatures were monitored with thermometers on the same shelves on which the plants were grown. All parental strains are available from the *Arabidopsis* Biological Resource Center at Ohio State University (Columbus, OH). All FTL lines are available upon request from G.P.C.

T-DNA Vector Construction. Plasmids carrying genes encoding the fluorescent proteins enhanced YFP (EYFP), enhanced CFP (ECFP), and Ds-Red (XFP collectively) were obtained from BD Bioscience Clontech (Palo Alto, CA). The ORFs for these marker proteins were transferred into the pENTR Gateway vector (Invitrogen, Carlsbad, CA) by PCR-based TOPO cloning using the manufacturer's instructions. The pENTR-based clones were used to transfer the marker genes into the pK2GW7 (kanamycin selection) or pB7WG2 (glufosinate selection) Gateway vectors that had been modified by removing the 35S promoter and replacing it with the pollen-specific postmeiotic LAT52 promoter (12). The transfer was accomplished by using the Invitrogen LR Clonase kit following the manufacturer's instructions.

Plant Transformation. *Agrobacterium tumefaciens* strain GV3101 was transformed with the LAT52:XFP T-DNA vectors by using electroporation. Plants were transformed by using the method of Chung *et al.* (23), except that the bacteria were resuspended in simplified media consisting of 5% sucrose and 0.05% Silwet L-77. Positive T_1 transformants harboring an *npt*II-based construct were selected on 0.5× MS salt medium containing 50 $mg \cdot ml^{-1}$ kanamycin after 2 weeks of growth and then transferred into soil. Bar-based T_1 transformants were germinated on soil and selected by spraying with 0.0189% glufosinate ammonium from Bayer Crop Science (Research Triangle Park, NC), sold under the name Liberty. Expression of the fluorescent marker proteins in the pollen was confirmed by using epifluorescence microscopy.

Ligation-Mediated Suppression PCR. The genomic location of each insertion was established by using LMS-PCR as described by Alonso *et al.* (13) with the following modifications. DNA from two to three cauline leaves was purified as described in Copenhaver *et al.* (24) and digested with either HindIII or EcoRI (NEB, Beverly, MA). Digested genomic DNA was chloroform-extracted, ethanol-precipitated, and suspended in 20 μl of double-distilled H_2O. Adapters were then ligated to the library of genomic fragments by using T4 ligase (NEB). Adapters for ligation to HindIII ends were made by annealing ADAPS-H3 with a 3′ N-terminal end with (acgtcacctgcccgg/3AmMc7) ADAPL-E1 (ctaatacgactcactatagggctcgagcggccgcccgggcaggtg). Adapters for ligation to EcoRI ends were made by annealing ADAPS-E1 with a 3′ N-terminal end (aattcacctgcccgg/3AmMc7) with ADAPL-E1. Primary PCR products were generated by using primers AP1 (ggatcctaatacgactcactataggc) and PgwLat52LB-WP1 (ctatgttactagatcgaccgg). Secondary PCR products were generated by diluting primary products 50-fold and amplifying with primers AP2 (tatagggctcgagcggccg) and PgwLat52LB-WP2 (caattcggcgttaattcagtac). Primary and secondary PCRs were initiated at 94° for 2 min followed by 29 cycles of 30-s denaturation (94°C), 30-s annealing (55°C), 1-min extension (72°C), and a final 10-min 65°C incubation. All primers were purchased from Integrated DNA Technologies (Skokie, IL).

PCR products were sequenced at the University of North Carolina Lineberger Comprehensive Cancer Center.

Mutagenesis. Nonfluorescent alleles of transgenes encoding fluorescent proteins were generated by following the protocol of Weigel and Glazebrook (25). Seeds (0.5 g) were imbibed in 30 ml of sterile water for 4 h and then mutagenized with 0.2% ethyl methane sulfonate for 16 h at room temperature with gentle agitation. Mutagenized seeds were rinsed with 30 ml of sterile water eight times, and then they were dried before planting.

Microscopy. Segregation patterns of fluorescent alleles in pollen tetrads were measured by using a Nikon (Melville, NY) E1000 epifluorescence microscope equipped with filters from Chroma Technology (Rochester, NY). Pollen was collected by dipping flowers into a drop of 0.1% Triton X-100 on a glass slide. Micrographs were taken with a Nikon Coolpix5000 camera. Multicolor images were obtained by using Adobe Photoshop (Adobe, San Jose, CA) to merge two or more single-color images taken by using the appropriate filter.

Linkage Analysis. To measure the map distance between any two transgenic markers, tetrads were designated parental ditype, NPD, or tetratype (T), depending on the segregation pattern of the marker pair. Map distances were then determined by using the Perkins formula: $100[(1/2\ T + 3\ NPD)/n]$ (26). Expected NPD frequencies for single intervals were calculated by using the Papazian formula: $1/2[(1-T)-(1-(3\ T/2))^{2/3}]$ (15).

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