

Traffic Lines: New Tools for Genetic Analysis in *Arabidopsis thaliana*

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ABSTRACT Genetic analysis requires the ability to identify the genotypes of individuals in a segregating population. This task is straightforward if each genotype has a distinctive phenotype, but is difficult if these genotypes are phenotypically similar or identical. We show that *Arabidopsis* seeds homozygous or heterozygous for a mutation of interest can be identified in a segregating family by placing the mutation in *trans* to a chromosome carrying a pair of seed-expressed green and red fluorescent transgenes (a “traffic line”) that flank the mutation. Nonfluorescent seeds in the self-pollinated progeny of such a heterozygous plant are usually homozygous for the mutation, whereas seeds with intermediate green and red fluorescence are typically heterozygous for the mutation. This makes it possible to identify seedlings homozygous for mutations that lack an obvious seedling phenotype, and also facilitates the analysis of lethal or sterile mutations, which must be propagated in heterozygous condition. Traffic lines can also be used to identify progeny that have undergone recombination within a defined region of the genome, facilitating genetic mapping and the production of near-isogenic lines. We produced 488 transgenic lines containing single genome-mapped insertions of *NAP:dsRED* and *NAP:eGFP* in Columbia (330 lines) and Landsberg *erecta* (158 lines) and generated sets of traffic lines that span most regions of the *Arabidopsis* genome. We demonstrated the utility of these lines for identifying seeds of a specific genotype and for generating near-isogenic lines using mutations of *WUSCHEL* and *SHOOTMERISTEMLESS*. This new resource significantly decreases the effort and cost of genotyping segregating families and increases the efficiency of experiments that rely on the ability to detect recombination in a defined chromosomal segment.

KEYWORDS recombination; *wus*; *stm*; T-DNA; mapping

ARABIDOPSIS *thaliana* has had a major impact on plant biology over the past 20 years because of the advantages it offers for genetic analysis (Somerville and Koornneef 2002; Koornneef and Meinke 2010). The ease of performing large-scale phenotype-based genetic screens, the availability of sequence-indexed mutations in most genes (Sundaresan *et al.* 1995; Budziszewski *et al.* 2001; McElver *et al.* 2001; Alonso *et al.* 2003), and techniques for site-specific mutagenesis and gene silencing (Chuang and Meyerowitz 2000; Alvarez *et al.* 2006; Schwab *et al.* 2006; Franco-Zorrilla *et al.* 2007; Todesco *et al.* 2010; Cermak *et al.* 2011) have all contributed to the

popularity of *Arabidopsis* as a model system in plant biology. But, although there are many ways to obtain mutations in genes of interest in *Arabidopsis*, there are surprisingly few ways to efficiently manipulate these mutations. Unless a mutation has an obvious, fully penetrant and viable phenotype, or is associated with a selectable marker, the only way it can be followed in genetic crosses is by molecular genotyping or progeny testing. Mutations that have invisible, weak, or variable phenotypes, as well as mutations that cannot be maintained under homozygous conditions (*e.g.*, lethal or sterile mutations), are difficult to work with. A tool that makes it possible to unambiguously identify plants homozygous or heterozygous for these types of mutations—without the need for molecular genotyping—would significantly enhance the efficiency of genetic analysis in this important experimental system.

In *Drosophila melanogaster*, this problem is solved with balancer chromosomes (Ashburner *et al.* 2005). Balancer

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chromosomes contain multiple inversions and are marked with a dominant, viable, mutation/transgene and a recessive lethal mutation. These features ensure that chromosomes in *trans* to the balancer are transmitted intact and provide a convenient way to maintain stocks of lethal or sterile mutations. Although balancer chromosomes exist in other species (Zheng *et al.* 1999; Wilson *et al.* 2005; Edgley *et al.* 2006), they have not been widely used outside of *Drosophila* because of the technical difficulty of generating such chromosomes.

Here we describe an approach that has some of the features of a balancer chromosome, but does not require extensive chromosome engineering. This approach was originally developed to study recombination in *Arabidopsis* and involves using a pair of linked, differently colored fluorescent transgenes to mark a chromosome interval (Melamed-Bessudo *et al.* 2005; Berchowitz *et al.* 2007; Francis *et al.* 2007; Berchowitz and Copenhaver 2008; Berchowitz and Copenhaver 2009; Pecinka *et al.* 2011; Melamed-Bessudo and Levy 2012). The first use of this approach (Melamed-Bessudo *et al.* 2005) employed fluorescent reporters (*eGFP* and *dsRED*) fused to the seed-specific *napA* storage protein promoter (Stuitje *et al.* 2003). We recognized that in addition to revealing the products of recombination, these doubly marked chromosomes provide a convenient way to follow nonrecombinant chromosome segments in genetic crosses. When *NAP:eGFP* and *NAP:dsRED* are linked on a single chromosome, most of the individuals that inherit a doubly marked chromosome from a parent hemizygous for these transgenes (*NAP:eGFP NAP:dsRED/-*) will possess a nonrecombinant version of the chromosome segment flanked by these two transgenes, and most of the individuals that lack *NAP:eGFP* and *NAP:dsRED* will contain a nonrecombinant version of the segment in *trans* to these markers; the exceptions are cases in which double recombination has occurred within the marked interval, and these will be rare for small intervals. This means that mutations present on the chromosome in *trans* to a doubly marked chromosome can be followed in genetic crosses by screening for nonfluorescent seeds in the progeny of heterozygous plants. Furthermore, because the intensity of *eGFP* and *dsRED* fluorescence is sensitive to gene dose, seeds heterozygous for a mutation within the marked interval can be distinguished from seeds homozygous for the *NAP:eGFP-NAP:dsRED* chromosome by their intermediate level of fluorescence. These properties are particularly useful for mutations with weak or invisible phenotypes, which are typically identified in segregating families by PCR genotyping or progeny testing and for mutations that cannot be maintained in homozygous condition, such as lethal or sterile mutations.

Transgenic lines containing *NAP:eGFP* or *NAP:dsRED* at a single site in the *Arabidopsis* genome were screened to identify lines that had no obvious homozygous phenotype, and the position of these transgenes was then determined by DNA sequencing. Linked *NAP:eGFP* and *NAP:dsRED* insertions were then joined by recombination to create a series of traffic lines (TLs) that span most of the *Arabidopsis*

genome. This new collection of marker lines and examples of the ways in which they can be used for genetic analysis are described in this article.

Materials and Methods

Vector construction

Plasmid pFLUAR100 harboring *NAP:eGFP*, and plasmid pFLUAR101 harboring *NAP:dsRED* were kindly provided by Dr. Antoine R. Stuitje (Department of Genetics, Institute for Molecular Cell Biology, The Netherlands). Due to the low genetic transformation frequencies associated with these two constructs and the low recovery rate of T-DNA border sequence, these constructs were modified using overlapping PCR and the high-fidelity Phusion DNA polymerase (NEB). For the pCAM-NAP:eGFP construct, the *napA* promoter sequence was PCR amplified using the forward primer 5'-CCCAAGCTTAGGAAGCTTGCATGCTGCAG-3' and the reverse primer 5'-TCCTCGCCCTTGCTCACCATGGGGTATGTTTTTAATCTTG-3', and the *eGFP* open reading frame was PCR amplified using the forward primer 5'-ATGGTGAGCAAGGGCGAGGA-3' and the reverse primer 5'-TGCCA AATGTTTGAACGATCTTACTTGTACAGCTCGTCCA-3'. The *nos* sequence was PCR amplified using the forward primer 5'-GATCGTTCAAACATTTGGCAATAAAG-3' and the reverse primer 5'-CCGCTCGAGGATCTAGTAACATAGATGACAC-3' and was used as the terminator for both the pCAM-NAP:eGFP and pCAM-NAP:dsRED constructs. For the pCAM-NAP:dsRED construct, the *napA* promoter sequence was PCR amplified using the forward primer 5'-CCCAAGCTTAGGAAGCTTGCATGCTGCAG-3' and the reverse primer 5'-ACAT TCTTGAAGACCGCATGGGGTATGTTTTTAATCTTG-3', and the *dsRED* open reading frame was PCR amplified using the forward primer 5'-ATGCGGTCTTCCAAGAATGT-3' and the reverse primer 5'-TGCCAAATGTTTGAACGATCTTAAAGGAACAGATGGTGGC-3'. After overlapping PCR, the *NAP:eGFP-NOS* expression cassette was cloned into the *XhoI* and *EcoRI*-linearized CAMBIA3300 vector, and the *NAP:dsRED-NOS* expression cassette was cloned into the *XhoI* and *BamHI*-linearized CAMBIA3300 vector. The resulting pCAM-NAP:eGFP and pCAM-NAP:dsRED plasmids were sequenced before they were electroporated into *Agrobacterium* competent cells for genetic transformation.

Selecting transgenic plants

Transformation was carried out by floral dipping (Clough and Bent 1998). Mature T1 seeds were screened using a Leica MZ FLIII stereomicroscope and long-bandpass GFP (ET480/40x/ET510 LP) and RFP (ET546/10x/ET590 nm LP) filter sets. It is also possible to identify transgenic seeds using the stereomicroscope adaptors available from Nightsea, Inc. (<http://www.nightsea.com/galleries/arabidopsis/>). Seeds were manually classified as low, medium, or high fluorescence and planted individually in 96 well flats. T1 plants were subsequently screened for semisterility and

embryo lethal mutations by opening immature siliques and looking for low seed set or defective seeds. Siliques with ~50% aborted seeds were classified as semisterile, whereas siliques with ~25% albino seeds were classified as having an embryo lethal mutation. Mature T2 seeds from fully fertile plants were screened with a stereomicroscope to identify families segregating 3:1 fluorescent:nonfluorescent seeds, and several of the brightest seeds from these families were planted to obtain lines homozygous for the insertion. If all of the homozygous plants appeared phenotypically normal, the sequence of the insertion site was determined.

Mapping transgenes

Insertion sites were sequenced using an adapter ligation-mediated PCR method as described previously (O'Malley *et al.* 2007) with some modifications. Genomic DNA was prepared by phenol-chloroform extraction. About 100 ng of genomic DNA was digested overnight at room temperature with *Hind*III and *Eco*RI in a cocktail containing 10× NEB ligase buffer, 1 μl. *Hind*III adapter, *Eco*RI adapter, and NEB T4 DNA ligase. Digested genomic DNA was used as the template in a 20-μl system for the first-round PCR with an adapter primer, 5'-GTAATACGACTCACTATAGGGC-3', and a NAPIN-NOS-LB1 primer, 5'-CCTGTTGCCGGTCTTGCGATGATT-3'. PCR was carried out using TAKARA Ex Taq Hot Start DNA polymerase as follows: predenaturation at 95° for 3 min, and then 29 cycles of 98° for 10 sec, 60° for 20 sec, 72° for 2 min and 30 sec, and extension at 72° for 7 min. PCR products were diluted with ddH₂O to 100 μl. Diluted PCR products, 1 μl, was used as the template for a second-round PCR with a AP2-C primer, 5'-TGGTCGACGGCCCGGGCTGC-3', and a NAPIN-NOS-LB2 primer, 5'-GCGCGGTGTCATCTATGTTAC TAG-3'. The second round of PCR was done as described above except that the annealing temperature was increased to 62°. PCR products were gel purified and sequenced, and the T-DNA border sequences were then mapped to the *Arabidopsis* genome using the SIGnAL T-DNA express mapping tool (<http://signal.salk.edu/cgi-bin/tdnaexpress>).

Stock distribution

The transgenic lines described in this article may be obtained from the *Arabidopsis* Biological Resource Center (ABRC) (<https://abrc.osu.edu>). ABRC stock numbers and other information about individual lines are available in the supporting information (Table S1, Table S2, Table S3, and Table S4), and online at <https://sites.sas.upenn.edu/poethig-lab/pages/data>. Complete sets of the lines described in this article are available from ABRC using the following set numbers: CS71568 (CG and CR lines), CS71569 (LG and LR lines), CS71570 (CTL lines), and CS71571 (LTL lines).

Results

Lines transformed with *NAP:eGFP* and *NAP:dsRED* were initially produced using the pFLUAR vectors (Stuitje *et al.* 2003). Consistent with a previous report (Melamed-Bessudo

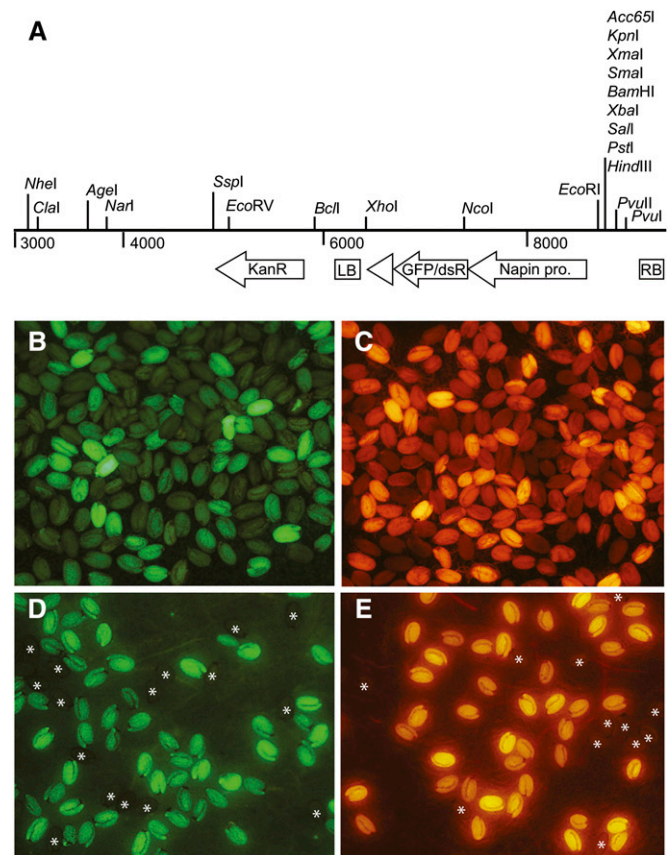


Figure 1 Dosage-sensitive expression of *NAP:eGFP* and *NAP:dsRED* in dry seeds. (A) Structure of the T-DNA vectors (pCAM–*NAP:eGFP* and pCAM–*NAP:dsRED*) used in this study. (B) T1 seeds transformed with *NAP:eGFP*. (C) T1 seeds transformed with *NAP:dsRED*. (D) T2 seeds from a transgenic plant segregating 3:1 for *NAP:eGFP*. (E) T2 seeds from a transgenic plant segregating 3:1 for *NAP:dsRED*. Nonfluorescent seeds are marked with an asterisk.

et al. 2005), these vectors had low transformation efficiency and their insertion sites were difficult to sequence. To solve these problems, we cloned *NAP:eGFP* and *NAP:dsRED* fragments from pFLUAR100 and pFLUAR101 into pCAM-BIA3300. These new vectors, pCAM–*NAP:dsRED* and pCAM–*NAP:eGFP* (Figure 1A), had significantly higher transformation efficiencies than the pFLUAR vectors, and we were able to determine the insertion site of the transgene in >80% of the lines we sequenced.

The fluorescence intensity of primary transformed seed (T1 seed) varied widely (Figure 1, B and C). To explore the basis for this phenomenon, Columbia (Col) seeds transformed with *NAP:eGFP* were divided into low, medium, and high fluorescence classes, and the number of insertion sites in these seeds was determined by counting the frequency of fluorescent and nonfluorescent seed in their T2 progeny. T1 plants that produced 3:1 fluorescent:nonfluorescent seeds were classified as having a single insertion site, whereas plants that produce >3:1 ratio of fluorescent:nonfluorescent seed were categorized as having more than one insertion site (Table 1). Only 18% of the T1 seeds in the high-fluorescence class contained a single insertion. In

Table 1 eGFP fluorescence intensity predicts transgene copy number

Fluorescence intensity of T1 seed	Frequency of single insertion lines
High	6/33 (18%)
Medium	25/41 (61%)
Low	31/45 (69%)

contrast, 60–70% of T1 seeds with low or medium fluorescence had a single insertion site. This result is consistent with previous studies indicating that plants transformed with *Agrobacterium tumefaciens* often contain T-DNA insertions at multiple sites (McElver *et al.* 2001; Alonso *et al.* 2003) and demonstrates that it is possible to enrich for plants containing single T-DNA insertions using seed fluorescence as a transformation marker.

The seeds of plants segregating for a single insertion usually varied in fluorescence intensity (Figure 1, D and E). In a previous study of a single transgenic line containing GFP under the regulation of the seed-specific *OLE1* promoter, it was reported that fluorescence intensity was correlated with homo- or hemizyosity for the transgene (Shimada *et al.* 2010). To determine if fluorescence intensity can indeed be used to determine the genotype of a seed, 10–15 bright fluorescent seeds from 52 independently derived T1 lines segregating 3:1 for *NAP:eGFP* were planted. The seeds produced by these 643 T2 plants were then examined to determine if the plant was homozygous for the transgene. In 95% of cases (610/643), the T2 plant was homozygous for the transgene. This result demonstrates that fluorescence intensity can be used to identify seeds homozygous for a T-DNA insertion.

Marker lines expressing *NAP:eGFP* and *NAP:dsRED*

The primary goal of this study was to develop a large set of transgene insertions useful as genetic markers. For this purpose, T1 seeds expressing moderate to low levels of eGFP and dsRED were planted, and their T2 seeds were examined to identify lines segregating 3:1 fluorescent:nonfluorescent seeds. Analyses of the first 40 of these lines revealed that 4 were semisterile when the transgene was hemizygous, but completely fertile when it was homozygous; these lines also displayed reduced recombination in the vicinity of the transgene. This behavior is characteristic of reciprocal translocations or large inversions and supports a previous study indicating that *Agrobacterium*-mediated transformation frequently produces large chromosomal rearrangements (Clark and Krysan 2010). Because reciprocal translocations make it difficult to use these lines for genetic mapping, immature siliques of T1 plants were screened to identify those with 50% undeveloped ovules (semisterile) or 25% aborted seed (embryo lethal mutation). Of T1 plants (Col), 16% were semisterile (Table 2), which is similar to the frequency of reciprocal translocations (19%) in the SALK T-DNA collection (Clark and Krysan 2010). Of T1 plants (Col), 54% were fully fertile and produced T2 families seg-

Table 2 Characterization of T2 families transformed with *NAP:eGFP* and *NAP:dsRED*

Transgene	Fertile			
	3:1 ^a	>3:1 ^b	Semisterile ^c	Embryo lethal ^d
<i>NAP:eGFP</i>	175 (57%)	74 (24%)	45 (15%)	14 (4%)
<i>NAP:dsRED</i>	140 (50%)	77 (28%)	48 (17%)	13 (5%)
Total	315 (54%)	151 (26%)	93 (16%)	27 (5%)

^a Number of completely fertile T2 families segregating 3:1 fluorescent:nonfluorescent seeds.

^b Number of completely fertile T2 families segregating greater than 3:1 fluorescent:nonfluorescent seeds.

^c Number of T2 families with 50% ovule abortion.

^d Number of T2 families segregating 25% aborted seed.

regating fluorescent and nonfluorescent seeds in a 3:1 ratio (Table 2). Three highly fluorescent seeds from these families were planted to obtain plants homozygous for the transgene. This also enabled us to identify insertions associated with a mutant phenotype, which was apparent when all three plants had the same aberrant phenotype. The insertion site in phenotypically normal families was amplified by adaptor-ligation PCR (O'Malley *et al.* 2007), gel purified, and sequenced. To date, we have determined the genomic locations of 330 *NAP:eGFP* and *NAP:dsRED* transgene insertions in Col (Figure 2A and Table S1) and 158 transgene insertions in Landsberg *erecta* (*Ler*) (Figure 2B and Table S2).

The recombination distance between linked *NAP:EGP* and *NAP:dsRED* transgenes was estimated using high-resolution mapping data from recombinant inbred lines (Singer *et al.* 2006). Lines containing insertions <30 cM apart were intercrossed, and the resulting hybrids were crossed as pollen parents to the appropriate parental ecotype (Col or *Ler*) to obtain recombinant chromosomes containing both transgenes (Figure 3). Stocks homozygous for the recombinant chromosome were produced by selecting brightly fluorescent seed from the F2 progeny of recombinant plants. We generated 74 recombinant lines in Col (Figure 4A and Table S1) and 26 recombinant lines in *Ler* (Figure 4B and Table S2), which cover most of the *Arabidopsis* genome. These stocks are named traffic lines (TLs) because their seeds fluoresce red, green, and yellow under appropriate illumination.

Using TLs to identify genotypes in segregating populations

Chromosome segments bounded by *NAP:eGFP* and *NAP:dsRED* are useful because both the marked segment, and the segment in *trans* to the marked chromosome, can be followed in genetic crosses: self or outcross progeny that inherit the marked chromosome will have green and red fluorescent seeds, whereas progeny that inherit the non-transgenic chromosome will have nonfluorescent seeds. Because double recombination within a short chromosome segment is rare, the vast majority of these nonfluorescent seeds will contain a parental version of the region in *trans* to the marked interval (Figure 5A). Additionally, the dosage sensitivity of *NAP:eGFP* and *NAP:dsRED* makes it possible to distinguish F2 seeds that are heterozygous for the marked

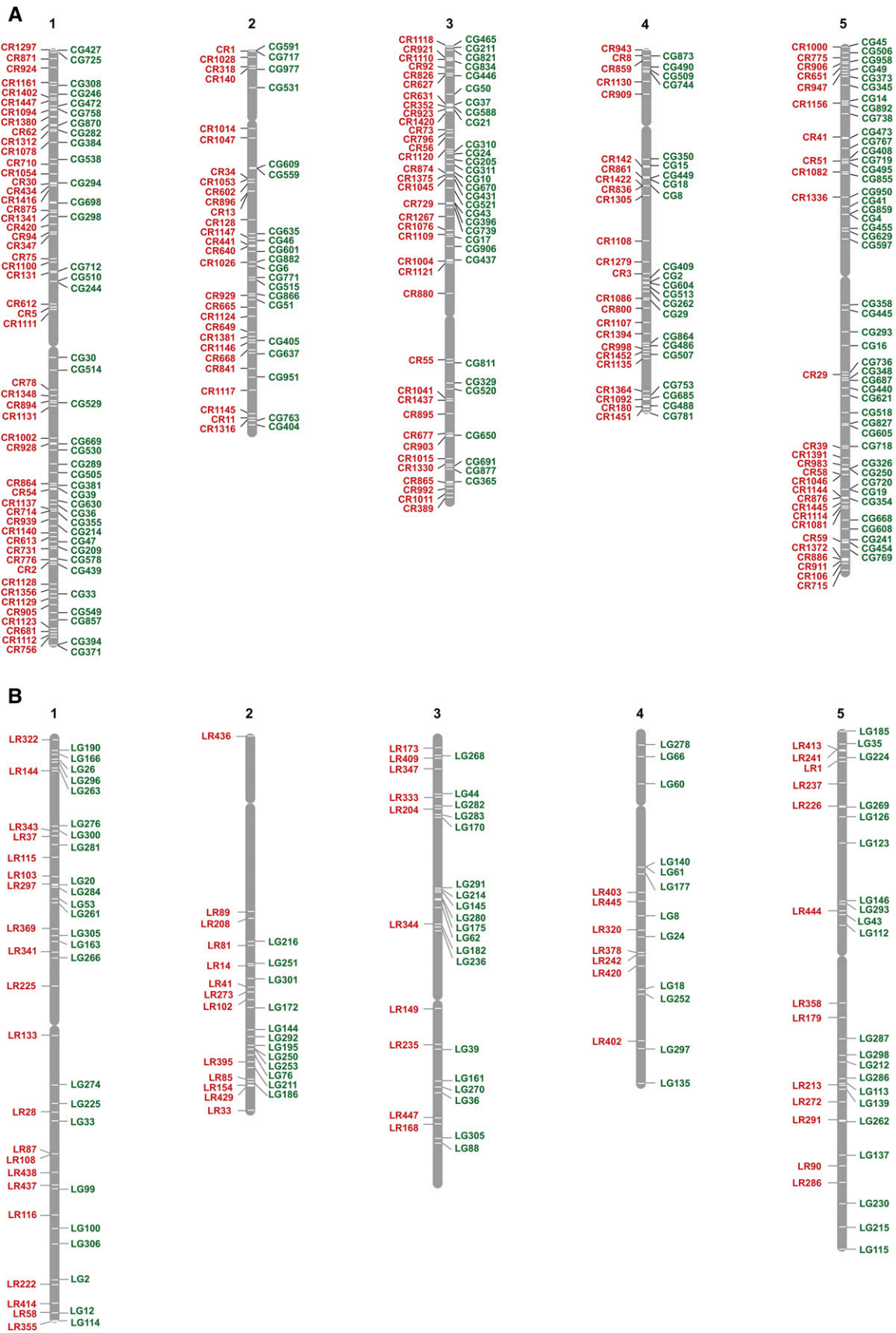


Figure 2 Genomic locations of *NAP:eGFP* and *NAP:dsRED* insertions. (A) Transgene insertions in Col. (B) Transgene insertions in Ler. Maps were

chromosome from seeds homozygous for this chromosome by their fluorescence intensity (Figure 5, A and B).

To test the value of TLs as a genotyping tool, we used them to identify seeds homozygous or heterozygous for *wuschel-5* (*wus-5*) (Sonoda *et al.* 2007; Chatfield *et al.* 2013) and *shootmeristemless-1* (*stm-1*) (Barton and Poethig 1993). *wus-5* is caused by a T-DNA insertion (SAIL_150_G06) and is identifiable in homozygous condition by its shoot defective phenotype and in heterozygous condition by the Basta-resistance conferred by the transgene insertion. *stm-1* is an EMS-induced allele of *SHOOTMERISTEMLESS*. Both mutations produce defective shoot meristems and therefore cannot be maintained in homozygous condition. Three TLs encompassing *WUS*—CTL2.1, CTL2.3, and CTL2.4—were crossed to plants heterozygous for *wus-5*, and nonfluorescent and bright fluorescent seeds in the F2 progeny of TL/*wus-5* plants were selected (Figure 5B). One hundred percent of the nonfluorescent F2 progeny of CTL2.1/*wus-5* and CTL2.4/*wus-5*, and 99% of the nonfluorescent F2 progeny of CTL2.3/*wus-5*, were homozygous for *wus-5* (Table 3). All of the plants from bright fluorescent seeds were morphologically normal (Table 3) and most were homozygous for the wild-type *WUS* allele, as evident from their sensitivity to Basta (Figure 5D). Similar results were obtained with *stm-1*. Plants heterozygous for *stm-1* were crossed to CTL1.4 and CTL1.9, and nonfluorescent F2 seeds of CTL/*stm-1* individuals were planted. All of these nonfluorescent seeds produced seedlings with defective meristems, indicating that they were homozygous for *stm-1* (Table 3). These results demonstrate that TLs can be used to identify seeds homozygous for a mutation located within the marked interval. To determine if TLs can be used to identify seeds heterozygous for a mutation, we selected seeds with intermediate red and green fluorescence in the F2 progeny of CTL2.4/*wus-5* (Figure 5B). Of these seeds 100% (90/90 plants) were phenotypically wild type and resistant to Basta, indicating that they were heterozygous for *wus-5*.

Single *NAP:EGFP* or *NAP:dsRED* insertions can also be used to identify seeds of a desired genotype if the insertion is tightly linked to the gene of interest. CR13 and CR128 contain *NAP:dsRED* insertions located, respectively, 600 kb above and 900 kb below *WUS* on chromosome 2 (Figure 4A). Ninety-six percent of the nonfluorescent seeds in the F2 progeny of CR13/*wus-5* and 94% of the nonfluorescent seeds in the F2 progeny of CR128/*wus-5* were homozygous for *wus* (Figure 5C; Table 3). Of the plants from seeds with intermediate fluorescence, 98% (84/86) were heterozygous for *wus-5*, as indicated by their morphologically normal, Basta-resistant phenotype.

It should be emphasized that distinguishing seeds homozygous and heterozygous for *NAP:eGFP* or *NAP:dsRED* can be

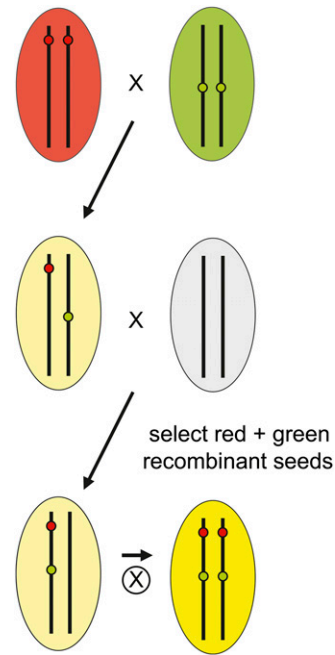


Figure 3 Crossing scheme used to produce TLs

difficult in some lines. Some transgenes produce similar levels of fluorescence in heterozygous and homozygous condition, whereas others produce variable fluorescence even when homozygous. Nevertheless, by selecting seeds at the ends of the fluorescence spectrum it is usually possible to reliably identify homozygous and heterozygous genotypes in a segregating population.

Using traffic lines to select for recombination in a defined region

To test the value of TLs for generating near-isogenic stocks, we introgressed *stm-1* (in *Ler*) into *Col*, using the strategy illustrated in Figure 6. Plants heterozygous for *stm-1* were crossed to CTL1.13 (Figure 4A), and F1 progeny containing *stm-1* were backcrossed to *Col*. Seeds from this backcross were screened to identify recombinants expressing green, but not red fluorescence. Seven of these recombinants were genotyped for markers within the CR54–CG33 interval to identify individuals with recombination sites close to *stm-1*. One plant with a recombination site ~300 kb below *stm-1* was crossed to CR54. The CR54 + *stm-1* CG33 progeny of this cross were then crossed to *Col*, and red–green recombinants [CR54 (*stm-1*) CG33/– + –] were planted and screened for *stm-1* by PCR. Of 54 recombinants, 8 were CR54 *stm-1* CG33/– + –. Seeds with the brightest red and green fluorescence were selected from the F2 progeny of these plants in order to obtain plants homozygous for the CR54 *stm-1* CG33 chromosome. In contrast to the

generated using the TAIR chromosome map tool (<https://www.arabidopsis.org/jsp/ChromosomeMap/tool.jsp>). CG and CR, *NAP:eGFP* and *NAP:dsRED* insertions in Columbia. LG and LR, *NAP:eGFP* and *NAP:dsRED* in *Ler*.

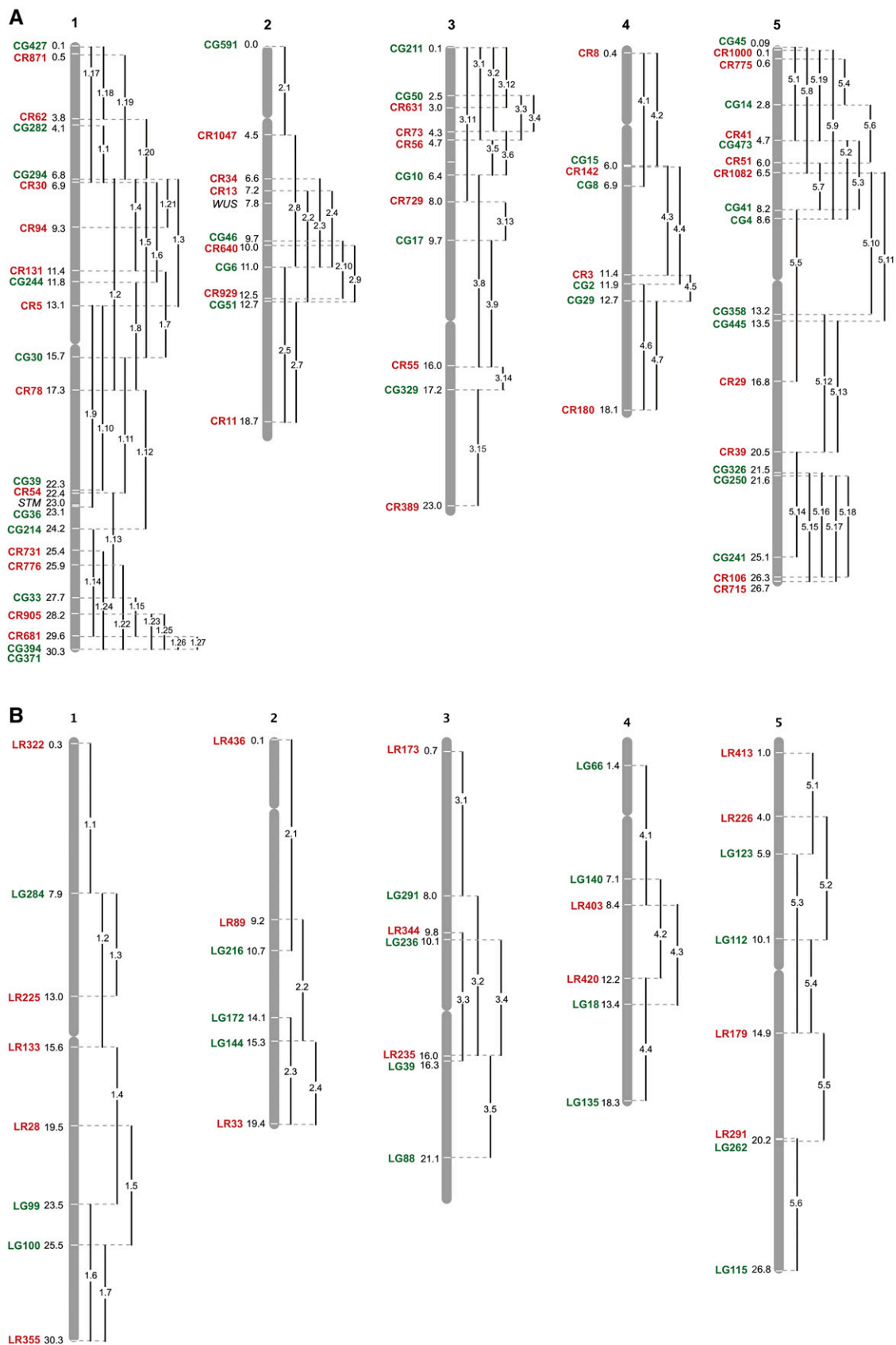


Figure 4 Location of *NAP:eGFP* and *NAP:dsRED* insertions in TLs. (A) TLs in Col; (B) TLs in Ler. Numbers indicate the nucleotide position of the insertion

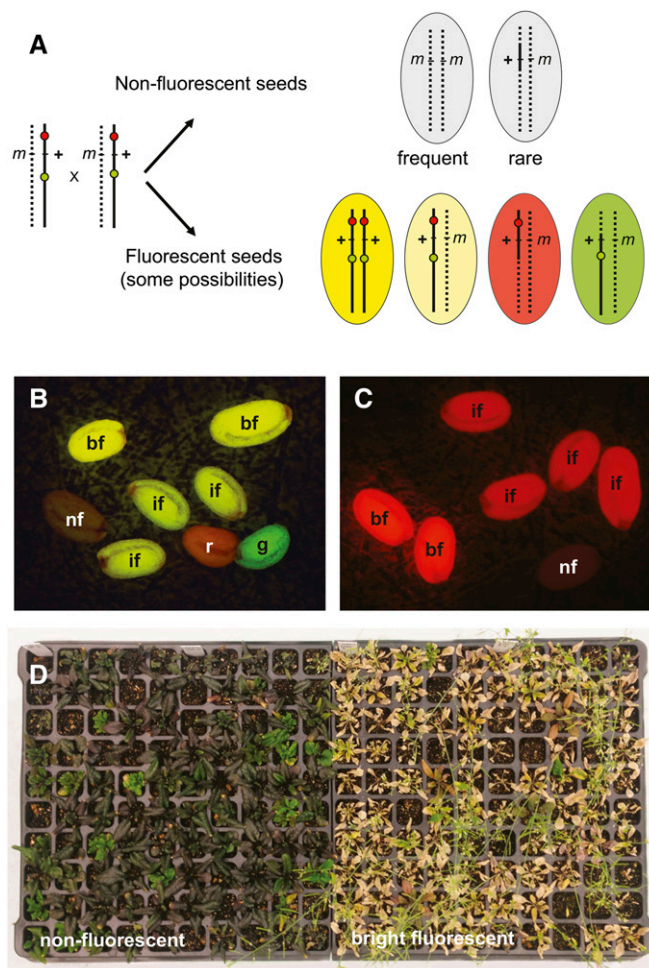


Figure 5 Using a TL to identify seeds homozygous for a recessive mutation. (A) The most common genotypes in the F2 progeny of a plant heterozygous for a TL and a mutation (*m*) of interest. (B) F2 seeds from a plant of the genotype CTL2.4/*wus-5* photographed using a broadband GFP filter set (see *Materials and Methods*), which allows GFP and dsRED fluorescence to be visualized simultaneously. (C) F2 seeds from a CR128/*wus-5* plant. nf, nonfluorescent; bf, bright fluorescent; if, intermediate fluorescent, r, red; g, green. (D) Phenotype of plants derived from non-fluorescent and bright fluorescent seeds in the F2 progeny of CTL2.4/*wus-5*. Plants were sprayed with Basta after flowering to determine the genotype of the phenotypically wild-type plants. All nonfluorescent seeds were resistant to Basta and had a *wus-5* mutant phenotype, indicating they were *wus-5/wus-5*, and most of the bright fluorescent seeds were homozygous for the wild-type *WUS* allele, as indicated by their sensitivity to Basta.

shootmeristemless phenotype of *stm-1* in a *Ler* background, these seedlings produced one or two rosette leaves from the primary axis and multiple defective axillary shoots; some plants produced an inflorescence with defective flowers (Figure 6). *stm-1* has a nonsense mutation upstream of the DNA binding domain of STM (Long *et al.* 1996) and consequently is likely to be a null allele. This result therefore implies that Col has one or more genes that can partially compensate for the loss of STM.

Discussion

Many problems in genetic analysis depend on the ability to determine the genotype of an individual independent of its phenotype. In *Arabidopsis*, this is usually done after the molecular nature of the relevant polymorphisms has been determined, so that they can be detected by a PCR-based technique. Although this approach is effective, it is time consuming and expensive and of course requires the destruction of at least a part of the individuals being genotyped. TLs make it possible to visually determine the genotype of a seed prior to planting, providing significant savings in time, space, and resources. They can be used either to propagate a chromosome segment intact in genetic crosses or to identify progeny resulting from recombination within a defined interval. These features enable a variety of experiments that are currently very difficult or impossible to perform in *Arabidopsis*.

The properties of plants transformed With NAP:eGFP and NAP:dsRED

TLs were generated using the seed-specific, semidominant fluorescent reporters, *NAP:eGFP* and *NAP:dsRED*. Aside from their value as genetic markers, these fluorescent reporters have several advantages over the herbicide or antibiotic resistance genes that are traditionally used for the identification of transgenic plants. Antibiotic and herbicide resistance are dominant traits, meaning that it is impossible to distinguish homozygous from hemizygous individuals on the basis of their resistance phenotype. In addition, these traits must be scored after germination, which requires both time and space. To identify a line homozygous for a single transgene using these dominant markers it is necessary to: (1) plant a large number of T1 seeds and treat with a selective agent to identify transgenic plants; (2) harvest T2 seeds from resistant plants and screen these families for lines segregating 3:1 for the selectable trait; (3) harvest seeds from five to six plants in families with single insertions; and (4) plant these T3 seeds to identify families homozygous for the insertion. In contrast, fluorescent reporters can be scored in dry seed (Stuitje *et al.* 2003; Lu and Kang 2008; Shimada *et al.* 2010; Untergasser *et al.* 2012) and are dosage sensitive (Shimada *et al.* 2010; this report). Using these markers, the procedure for identifying lines homozygous for single transgenic insertions consists of: (1) visually selecting transgenic T1 seeds prior to planting; (2) screening the F2 seed from these T1 plants to identify lines segregating 3:1 for the fluorescent transgene, and (3) planting a few (three to five) of the brightest seeds from these families to obtain homozygous lines. The phenotype of the plants produced by these bright seeds will also reveal whether the transgene has generated a recessive mutation; in this case, all of these seeds will produce plants with a mutant phenotype. Our observation

in megabases. Vertical lines connect the red and green transgenes in a TL. TLs are identified by the ecotype, chromosome, and the order in which the line was created (e.g., CTL1.2, Columbia, chromosome 1, second line).

Table 3 Phenotype of the F2 progeny of plants heterozygous for a TL or a single transgene

Parental genotype	Nonfluorescent F2 seeds			Bright fluorescent F2 seeds		
	Mutant ^a	Wild-type ^b	% mutant	Mutant ^a	Wild-type ^b	% wild-type
CTL2.1/ <i>wus-5</i>	92	0	100	0	74	100
CTL2.3/ <i>wus-5</i>	92	1	99	0	79	100
CTL2.4/ <i>wus-5</i>	91	0	100	0	76	100
CR128/ <i>wus-5</i>	116	7	94	0	37	100
CR13/ <i>wus-6</i>	139	6	96	0	82	100
CTL1.9/ <i>stm-1</i>	83	0	100	ND ^c	ND	ND
CTL1.14/ <i>stm-1</i>	93	0	100	ND	ND	ND

^a Number of seedlings displaying a *wus* or *stm* phenotype.

^b Number of phenotypically wild-type seedlings.

^c Not determined.

that seed fluorescence is correlated with the number of transgenic insertions also means that it is possible to enrich for T1 plants with single insertions prior to planting. Finally, it is worth noting that fluorescent proteins expressed under the regulation of the *napA* promoter are only visible late in seed development and for a short time following germination and thus do not interfere with the use of these proteins as gene expression markers. Seed-specific fluorescent markers such as *NAP:eGFP* or *NAP:dsRED* therefore provide enormous advantages for the generation and analysis of transgenic lines in plant biology. They are particularly useful for the molecular breeding of crop plants (Lu and Kang 2008) because they significantly reduce the time and space required to obtain homozygous transgenic lines in these large, relatively long-lived plants.

Although the *NAP:eGFP* and *NAP:dsRED* transgenes described in this study have many advantages, they are still susceptible to the problems inherent in T-DNA transformation. Consistent with previous studies (Szabados *et al.* 2002; Alonso *et al.* 2003), the insertion sites were distributed in a nonrandom fashion. Some regions had relatively few T-DNA insertions, whereas others were T-DNA insertion hotspots. Although this property makes it difficult to obtain even coverage of the genome, it is not a serious impediment to the use of these insertions as genetic markers because, as we show in this article, intervals that lack transgenes can be marked by a flanking pair of transgenes of different colors. The fluorescence intensity of lines containing a single T-DNA insertion site also varied quite significantly. This could reflect the presence of variable numbers of T-DNAs at each site, or the position of these T-DNAs in the genome. Whatever its basis, this phenomenon demonstrates the difficulty of generating transgenic lines with predictable levels of transgene expression. Many T1 plants were semisterile. Consistent with the conclusions of a previous study (Clark and Krysan 2010), we found that in most cases the transgene in these lines was completely fertile when homozygous, suggesting that it was associated with either a reciprocal translocation or a large inversion. We try to select against such chromosomal rearrangements by prescreening T1 lines for semisterility, but this approach does not detect small inversions, which are nearly completely fertile even in heterozy-

gous condition. Investigators interested in selecting for recombination in the vicinity of a specific transgene should be aware that some transgenes may be associated with a small inversion and guard against this possibility by using overlapping TLs. Finally, it should be emphasized that while we made an effort to select stably fluorescent lines, we have not propagated these lines for enough generations to predict their long-term stability; several TLs had to be discarded because one or both of the transgenes began to silence in advanced generations.

Some uses of traffic lines

TLs facilitate the use of segregating families for genetic analysis because seeds of a desired genotype can be accurately identified prior to planting. This is particularly important in the case of lethal or sterile mutations because these mutations must be propagated as heterozygotes; the ability to visually identify seeds heterozygous for these mutations in segregating families greatly reduces the effort required for stock maintenance. It is sometimes also important to identify plants homozygous for such mutations before the mutant phenotype becomes obvious (*e.g.*, for gene expression studies); TLs make it possible to collect large numbers of homozygous mutant seeds from a segregating family in a few hours. TLs are also useful for analyses of mutations with weak or environmentally sensitive phenotypes, as well as for phenotypic evaluation of mutations that exist in heterogeneous genetic backgrounds. In *Arabidopsis*, mutations are typically maintained as homozygous lines and compared to an independently maintained homozygous line of a different genotype. This approach requires that the lines being compared are isogenic for the genes of interest and also assumes that the relevant phenotype is insensitive to maternal influence or seed age effects. However, unless the mutation has been backcrossed to the wild-type control multiple times, the mutant line will differ from wild type at numerous sites in the genome, making it difficult to obtain meaningful phenotypic information. One way to avoid this problem is to compare the phenotype of mutant and wild-type siblings in a segregating family. Siblings share the same maternal environment and, on average, have the same genetic background because second-site mutations unlinked to

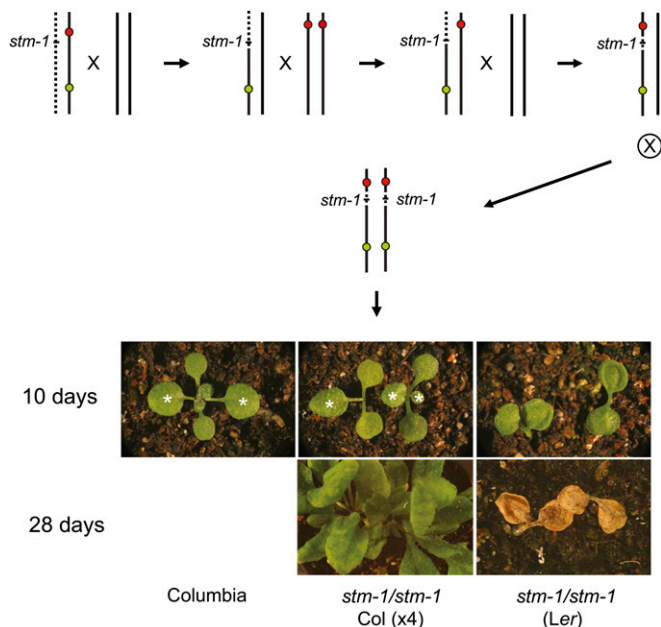


Figure 6 Using a TL to select recombinants in a specific chromosome segment. Procedure used to introgress *stm-1* into a Col genetic background. The transgenic chromosome is CTL1.13. Recombinants containing *stm-1* were identified by progeny testing or by molecular genotyping. *stm-1* has a much weaker phenotype in a Col background than in Ler. Col flowered 28 days after planting.

the mutation of interest will segregate randomly among both mutant and wild-type siblings. Although mutations closely linked to the mutation of interest will more often be homozygous in mutant than in wild-type plants, this approach is still a significant improvement over the use of independent mutant and wild-type lines for phenotypic analysis. TLs make it possible to identify seeds homozygous and heterozygous (or wild type) for a mutation of interest in a segregating family, so that the phenotypes of these genotypes can be compared.

TLs can also be used to detect and preserve the products of rare recombination events (Melamed-Bessudo *et al.* 2005; Shaked *et al.* 2006; Pecinka *et al.* 2011; Melamed-Bessudo and Levy 2012). This is particularly valuable for the creation of near-isogenic lines from mutagenized populations and for the analysis of quantitative trait loci (QTL). Newly identified mutations from a forward mutagenesis experiment must be backcrossed to the progenitor line several times to remove the second-site mutations present in the mutagenized line. Second-site mutations unlinked to the mutation-of-interest can be eliminated by five to six backcrosses. However, second-site mutations closely linked to the desired mutation are difficult to eliminate because crossing over in the vicinity of this mutation will be relatively rare. Furthermore, recombination in this region is difficult to detect because—with the exception of newly induced mutations—the mutagenized line is genetically identical to the progenitor line. To remove second-site mutations linked to a gene of interest, these mutations must be identified by sequencing the genome of

the mutagenized line and then selected against in a backcross population using PCR primers specific for the second-site mutation. TLs make it possible to detect recombination in the regions flanking a gene of interest, so that these regions can be removed without the need for information about any mutations present in these regions. TLs can also be used to introgress small chromosome segments from different accessions or ecotypes into a common genetic background, which is important for QTL analysis or for the purpose of comparing the phenotypes of mutations isolated in different genetic backgrounds.

Unlike traditional balancer chromosomes—which require extensive engineering—a fluorescent marker-based system can be easily developed. Because all major model genetic systems are capable of being transformed, and many different colored fluorescent markers are currently available, it should be straightforward to generate doubly marked strains using promoters appropriate for the system of interest. In principle, the approach described here could be used in any system to facilitate the maintenance and manipulation of useful genotypes.

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GENETICS

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Traffic Lines: New Tools for Genetic Analysis in *Arabidopsis thaliana*

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Tables S1-S4

Available for download as Excel files at <http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.173435/-/DC1>

Table S1 Genomic location of *NAP:eGFP* (CG) and *NAP:dsRED* (CR) insertions in transgenic lines of Columbia.

Table S2 Genomic location of *NAP:eGFP* (LG) and *NAP:dsRED* (LR) insertions in transgenic lines of Landsberg *erecta*

Table S3 Recombination distance between markers in Columbia traffic lines (CTL)

Table S4 Recombination distance between markers in Landsberg *erecta* traffic lines (LTL)