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Genetic Screens for Floral Mutants in *Arabidopsis thaliana*: Enhancers and Suppressors

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Abstract

The flower is a hallmark feature that has contributed to the evolutionary success of land plants. Diverse mutagenic agents have been employed as a tool to genetically perturb flower development and identify genes involved in floral patterning and morphogenesis. Since the initial studies to identify genes governing processes such as floral organ specification, mutagenesis in sensitized backgrounds has been used to isolate enhancers and suppressors to further probe the molecular basis of floral development. Here, we first describe two commonly employed methods for mutagenesis (using ethyl methanesulfonate (EMS) or T-DNAs as mutagens), and then describe three methods for identifying a mutation that leads to phenotypic alterations—traditional map-based cloning, TAIL-PCR, and deep sequencing in the plant model *Arabidopsis thaliana*.

Keywords

EMS; T-DNA; Floral development; Mutagenesis; Genetic screen; *Arabidopsis*; Map-based cloning; TAIL-PCR

1 Introduction

Plants, being sessile organisms, have to cope with many different stresses, both biotic and abiotic. The flower is a major contributing factor to the success and robustness of angiosperms. Until the 1980s, studies on plants had been largely limited to agriculturally important species; however, it is often difficult to work with these plants due to their large, in some cases, polyploid genomes. The small weed *Arabidopsis thaliana* was first purported as a genetic model system for plants by Friedrich Laibach [1]. *Arabidopsis* is amenable to genetic analyses due to its small size, small genome (125 Mb), rapid generation time (5–6 weeks from seed to seed), high fecundity (up to 10,000 seeds per plant), low repetitive sequences, and the ability to self-fertilize [1–5]. The power of *Arabidopsis* as a model system was evidenced, in part, by the identification of a series of genes involved in floral development in the late 1980s and early 1990s [6–14]. Since then, *Arabidopsis* has been the genetic model organism for plants and has been used to study diverse biological pathways.

Chemical mutagenesis has been used extensively to genetically perturb biological pathways in *Arabidopsis* in order to identify components of these pathways. Initial genetic screens are

often conducted in a wild-type background to identify major, nonredundant factors. However, such screens often fail to identify regulatory genes that have overlapping functions with other (often closely related) genes. Genetic modifier screens can be conducted to circumvent this genetic redundancy. A genetic modifier screen is performed in a mutant background, usually a weak allele in a major player in a biological process, in order to isolate other genes in that biological pathway. The second-site mutations resulting from this screen can either lead to an enhancement or to a suppression of the phenotype of the mutant plants that are being mutagenized.

There have been several notable examples in which modifier screens have allowed researchers to effectively dissect complex genetic pathways in floral development. For instance, *AGAMOUS* (*AG*) controls carpel and stamen development in *Arabidopsis*, and loss of *AG* results in floral patterning defects and a reiterative flower-in-flower phenotype indicative of loss of floral determinacy [7, 14, 15]. Many alleles of *AG* have been identified, ranging from plants with a weak (*ag-10*) to strong (*ag-1*) phenotype. One of the alleles, *ag-4*, generates a partially functional protein that is able to confer stamen identity, but floral determinacy or carpel identity is compromised [16]. Dr. Xuemei Chen in Dr. Elliot Meyerowitz's laboratory performed an EMS mutagenesis in the *ag-4* background and identified a mutant with an enhanced phenotype, reminiscent of *ag-1* (or flowers in which the stamens have been converted to petals). Map-based cloning revealed that the enhanced phenotype was due to mutations in two genes, which she named *HUA1* and *HUA2* (*HUA* means flower in Chinese) [17]. *HUA1* is an RNA-binding protein and *HUA2* is a novel protein involved in RNA processing [17–19]. *hua1 hua2* double mutants have a weak phenotype, while the single mutants are phenotypically normal [17]. Using the *hua1 hua2* double mutant, Dr. Chen and her group performed another EMS mutagenesis screen to identify mutants with enhanced floral determinacy and organ identity defects. From this screen, many mutants were isolated, including another weak allele of *AG*, *ag-10*; mutant alleles of *HEN4*, a gene encoding a KH domain RNA-binding protein; mutants in *HEN1*, a gene involved small RNA biogenesis; and a mutant in *ARGONAUTE10* (*AGO10*), an effector in microRNA function [20]. Studies with the *hua1 hua2 hen4* mutants revealed the redundant functions of the three genes in promoting the transcription elongation or splicing of *AG* pre-mRNA [18]. Subsequent work on *HEN1* eventually led to the discovery that miR172 promotes floral determinacy by repressing its target gene *APETALA2* (*AP2*) [21]. Furthermore, Dr. Xigang Liu, a postdoctoral scholar in Dr. Chen's laboratory, performed an enhancer screen using the weak *ag-10* allele. From that screen, *CURLY LEAF* and *AGO10* were among the few genes identified and shown to be involved in floral determinacy [20, 22]. Figure 1 depicts the discovery of players that specify the identities of the reproductive organs or confer floral determinacy through genetic screens.

Another well-known example of the usefulness of modifier screens involves the gene *CRABS CLAW* (*CRC*) and the pathway concerning polarity in *Arabidopsis* carpels. *CRC* is the founding member of the YABBY family of transcription factors, in which members are involved in establishing polar differentiation of lateral organs [23, 24]. Loss-of-function *crc* alleles were shown to be defective in carpel and nectary development, but none of the phenotypes in the mutant alleles indicated a defect in polar differentiation [15]. A modifier

screen of *crc* led to the identification of *PICKLE* (*PKL*) and *KANADII* (*KANI*), and the discovery that *CRC* promotes abaxial identity in *Arabidopsis* carpels. This function is normally masked by *KANI*, an abaxial identity promoting gene, and *PKL*, a gene that finely regulates meristematic activity [25]. Another modifier screen (using EMS) was done on *kan1-2 pkl-12* plants to search for other redundant genes in establishing carpel polarity. From this screen, four different enhancer loci were identified: *crc*, *hasty* (*hst*), *splayed* (*spl*), and *kanadi2* (*kan2*) [26–28].

Although most genetic screens in floral development have been used to identify enhancers, a good example of a suppressor screen is the identification of *CORYNE* (*CRN*) as an essential component of the stem-cell restricting CLAVATA3 (*CLV3*) signaling pathway [29]. Since increased *CLV3* signaling arrests meristem function leading to a facile phenotypic output, Muller and colleagues performed an EMS mutagenesis experiment with a mild *CLV3*-overexpressing line such that the meristem arrest is not that severe, so the plants are able to set seed [29, 30]. A mutant was isolated and found to be a *crn-1* allele, which has an aberrant silique shape reminiscent of *clv* mutants. Interestingly, the flowers in *crn-1* have enlarged gynoecium and some flowers have additional sepals or petals, probably caused by an increase in floral meristem size [29]. Initially, *CRN* was thought to be a receptor kinase that aids in the transmission of the *CLV3* signal [29]; however, it was later found to be a pseudokinase and is hypothesized to play a scaffolding role, perhaps to aid the export of *CLV2* to the plasma membrane and/or to assemble higher-order *CLV1* or *CLV1*-substrate complexes [31].

In addition to the three pathways mentioned above, modifier screens have been used for the isolation of mutants in *DORNRÖSCHEN-LIKE* from the *pi-5* background [32]; mutants in three *FUSED FLORAL ORGANS* genes from a *ufo* (*unusual floral organs*) allele [33]; and mutants in *SPLATED* (*STD*) from a weak *leafy* mutant background [27]. Thus, the aforementioned experiments highlight the power of modifier screens in teasing apart the molecular mechanisms governing different aspects of floral development. There have been several diverse reviews [34, 35] as well as protocols for EMS mutagenesis [36]; the present chapter therefore offers a comprehensive view of both EMS and insertional mutagenesis and outlines methods used to identify the mutation(s) responsible for the aberrant floral phenotypes. The chapter is divided into two parts. First, we provide facile protocols for two methods of mutagenesis using EMS and T-DNA as mutagens. Second, we describe three protocols used to identify the molecular lesion responsible for the mutant phenotypes.

1.1 Mutagenesis of *Arabidopsis*

Due to its small genome size, fecundity, and ease of transformation, *Arabidopsis* is the ideal organism to perform genetic mutagenesis screens. First, we discuss two major, divergent agents of mutagenesis: EMS and T-DNA.

1.1.1 EMS Mutagenesis of *Arabidopsis*—EMS is an alkylating agent that, ~90 % of the time, induces C/G to T/A substitutions [37, 38]. At a low frequency, EMS can also generate G/C to C/G or G/C to T/A transversions by 7-ethylguanine hydrolysis or A/T to G/C transition by 3-ethyladenine pairing errors [37]. In *Arabidopsis*, the frequencies of

EMS-induced stop codon and missense mutations are about ~5 % and ~65 %, respectively [39]. EMS mutagenesis allows the identification of loss- and gain-of-function mutants and can help researchers to understand the function of specific amino acids within a protein.

EMS mutagenesis tends to lead to plants containing more than one mutation (this issue will be addressed later). However, to ensure adequate coverage of the genome, meaning that one will get a mutation in every single gene, a large screening population must be obtained. A study showed that saturation could theoretically be achieved if 135,000M1 lines (five-fold coverage) are obtained [40]. In addition, the desired phenotypic output plays a role in saturation levels as well as the amount and time the seeds were exposed to EMS.

1.1.2 T-DNA Insertional Mutagenesis—Another common mode of mutagenesis is the use of *Agrobacterium-mediated* transformation via the floral dip method [41]. Transfer DNA (T-DNA) transformation is a phenomenon by which *Agrobacterium tumefaciens* inserts a portion of its Ti plasmid into the host (plant) genome, usually causing infection. Scientists have used this natural machinery and manipulated it in order to transfer their gene of interest or in cases of random mutagenesis, a selectable marker placed in lieu of the normal T-DNA, into *Arabidopsis*. The T-DNA region is flanked by two 25-bp repeat regions called the left border (LB) and right border (RB) (*see* Fig. 2). T-DNA integration occurs through illegal recombination by utilizing the plant DNA double-stranded break repair system. Several models of integration have been proposed; however, there is a consensus that integration is random and is mediated by the LB and RB [42]. T-DNA integration is higher at gene-rich regions than centromeric regions. Moreover, in actively transcribed genes, integration is higher around the transcription initiation and termination sites than in coding regions [43–45].

Both EMS and T-DNA insertional mutagenesis have been widely used as tools to probe diverse biological pathways in *Arabidopsis*. However, there are advantages as well as caveats for each technique. EMS mutagenesis is performed on seeds, making the procedure easy to perform; however, there is a low statistical probability that any one gene will be mutated in any one plant. Therefore, a copious amount of seeds need to be processed in each mutagenesis. Furthermore, the kill rate increases exponentially with dose, while the mutation rate for any single gene rises linearly. Hence, achieving saturation in a genetic screen is quite difficult. In addition, multiple mutations may incur within a single plant, so that backcrossing is necessary to ensure the elimination of background mutations unrelated to the phenotypes of interest. One advantage of EMS mutagenesis is that it can produce viable, weak alleles in genes whose function is essential to the plant. In contrast, T-DNA mutagenesis often produces loss-of-function mutants as integration of the T-DNA usually perturbs the gene's function. T-DNA mutagenesis can also be used to overexpress genes that are located close to the T-DNA insertion if the T-DNA harbors transcriptional enhancers [46]. An advantage of T-DNA mutagenesis compared to the use of EMS is that T-DNA insertion sites can often be readily identified using PCR-based approaches. A possible disadvantage is that it can result in chromosomal rearrangements, such as inversions or deletions. Moreover, multiple insertions and complex T-DNA loci may incur [47]. In addition, not all mutations generated through T-DNA transformation are actually caused by T-DNA insertion events [47]. Linkage studies or rescue experiments are necessary to

determine whether a mutant phenotype is caused by a T-DNA insertion. After mutagenesis and isolation of a mutant with the desired phenotype, the next step is to identify the mutation(s) responsible for the phenotype.

1.2 Identification of the Mutation Responsible for the Observed Mutant Phenotype

There are several experimental strategies that can be employed for the identification of a mutation, which causes a mutant phenotype. These include: map-based cloning (mainly used for EMS-induced mutations), TAIL-PCR (for T-DNA insertion mutants), as well as genome re-sequencing (for both EMS and T-DNA induced mutations).

1.2.1 Map-Based Positional Cloning—Map-based positional cloning is the most common method used to identify mutations after mutagenesis. It is a PCR-based method relying on markers, such as simple sequence length polymorphisms (SSLPs); cleaved amplified polymorphic sequences (CAPS); and derived CAPS (dCAPS). Many such markers have been described [48], but several new markers have been designed by our laboratory, which allow a better distinction between sequences derived from *Ler* and *Col-0* accessions (*see* Table 1). The underlying premise of this technique is to cross the mutant of interest into a different accession. In the F2 generation, in which the mutant phenotype is observed, one can determine where the mutation lies due to innate sequence differences between the accessions as markers in genetic mapping. This technique is often laborious and time-consuming. Theoretically, 300–400 plants are sufficient to narrow down the region where a mutated gene is located. However, in practice, thousands of plants may need to be processed.

1.2.2 Thermal Asymmetric Interlaced Polymerase Chain Reaction (TAIL-PCR)—One of the advantages of T-DNA insertional mutagenesis is that since the RB and LB sequences are known, TAIL-PCR can be performed to discover the genomic position of the T-DNA insertion. TAIL-PCR was first described in 1995 by Liu and Whittier [49]. In summary, TAIL-PCR utilizes nested primers in three consecutive reactions in order to find the insertion site [50]. Four initial PCR reactions are conducted, each with a “longer,” arbitrary degenerate (LAD) primer (one each from LAD1→4) in combination with primer R1. Products from these reactions are then utilized as a template in a second round of PCR reactions with a primer termed AC1 (AC1 partially overlaps with the LAD primers, except it does not contain the degenerate sequences) and the primer R2 from the T-DNA region (*see* Fig. 2 and Table 2). A third round of PCR reactions are conducted with products from the second round as templates and the AC1/R3 primer pair (*see* Fig. 2 and Table 2). The products from the second and third rounds of PCR are resolved in an agarose gel and products containing a single band *and* with the correct band patterning (the product from the third round of PCR should run faster than that from the second round of PCR) are sequenced. The advantages of TAIL-PCR are that it is a simple, sensitive, efficient, and fast method; that it has high specificity; and that the PCR product can be directly sequenced [49]. However, the major disadvantage is that this procedure will not work if multiple copies of the T-DNA are inserted in different positions in the genome, are tandemly inverted at the same position, or if the T-DNA is only partially inserted. Thus, it is important that genetic analysis be performed to ensure single locus insertion prior to performing TAIL-PCR.

However, this does not help if multiple copies are inserted within a single locus and/or the copies are inverted. Further, the vector backbone may also get transferred or the insertion may cause chromosomal rearrangements. In addition, there may not be linkage between the T-DNA and the phenotype, or the T-DNA may be partially inserted [47]. Thus, in these cases, map-based positional cloning or deep sequencing is utilized to identify the gene that, when mutated, leads to the observed phenotype.

1.2.3 Mapping Using Deep Sequencing Technology—As mentioned above, the map-based cloning method is commonly used to identify mutations causing a phenotype. However, there are some problems that prevent this method from being used in all cases. One, if the phenotype studied is very sensitive to the genetic background, then map-based cloning would not be applicable. For example, the phenotype may become suppressed when crossed to another accession, so that F2 mutant plants cannot be identified. Also, if the mutation occurs in a region where the recombination rate is low, thousands of plants will be needed to locate the mutation, which makes the process very tedious and time-consuming. With technological advances, another method is available to identify genes from mutagenesis experiments—high-throughput DNA sequencing. Though it is presently costly, with technological advances, this procedure should become more affordable.

The identification of EMS-induced mutations responsible for the observed phenotype can be accomplished in three manners using high-throughput sequencing. Firstly, bulk analyses of an F2 mapping population can be utilized by following the same rules as map-based cloning. DNA from pooled F2 plants of the mutant phenotype is used to produce a library for deep sequencing, which provides information on the segregation of various SNPs relative to the mutation. Linkage to certain SNPs helps to narrow down the region containing the mutation. Searching for genes with point mutations in this region then helps identify candidate genes. Two pipelines, SHOREMAP (<http://1001genomes.org/software/shoremap.html>) and NGM (<http://bar.utoronto.ca/NGM/>), have been developed to locate the mutation caused by EMS using bulk analysis of an F2 mapping population [51, 52]. It is suggested to use around ~500 plants when using SHOREMAP and around 80 plants when using NGM.

Secondly, bulk analysis of an F2 backcross population can also be used to identify the mutation in question. Locating the position of the mutation responsible for the mutant phenotype relies on the fact that other EMS-generated mutations in the genome segregate according to their linkage (or not) with the mutation in question. SHOREMAP provides an algorithm to locate a mutation using an F2 backcross population. To rule out the non-causal mutations, it is suggested to sequence pooled DNA from wild-type-looking plants as well [53].

Finally, the phenotype-causing mutation can also be found through deep sequencing of the mutant itself, which has already been used in our and several other laboratories [54]. Since the rough location of the mutation is not known, there may be too many candidates. Several practices may help to filter the candidates. Backcrosses prior to sequencing can reduce the number of non-causal mutations. Preliminary rough mapping information helps to limit the causal mutation to a region. Sequencing the parental line from which the mutant was derived

also helps to eliminate nonphenotype-causing mutations. Sequencing multiple mutant alleles in the same gene also helps pinpointing the gene of interest.

To identify the mutations caused by T-DNA transformation, there are two possible scenarios. For mutations caused by the T-DNA insertion, the affected gene could be easily located by the reads containing both genomic DNA and T-DNA sequences. To confidently identify such chimeric reads, it is better to sequence at 100 cycles (rather than at 50). For mutations caused by T-DNA transformation but that are not linked to a T-DNA insertion, the identification of candidate mutations is similar but more complicated than for EMS-generated mutations since three types of mutations: big INDELs (insertion and deletion), small INDELs, or SNPs (single nucleotide polymorphisms) need to be considered. To locate the region containing the mutation, the mapping can be done in the same manner as that for EMS-generated mutations. For the identification of the mutation from the region of interest, the three types of potential changes all need to be considered.

Using deep sequencing technology to find a mutation is not limited to the accessions with sequenced genomes. As long as the species has a reference genome, deep sequencing can be applied to mutants derived from un-sequenced accessions. Which methods to use and how to analyze the data depend on the situation and the specific experiment at hand. Despite these variables, the process of building the DNA sequencing libraries can be done in the same way, which will be described in this chapter.

2 Materials

2.1 Mutagenesis, Mutant Screening, and Initial Mapping of Mutations

2.1.1 EMS Mutagenesis of Arabidopsis—

1. 1 mL of Arabidopsis seeds (*see* Note 1).
2. Twenty trays of soil with 12 pots per tray.
3. Disposable 50 mL conical tubes.
4. Parafilm.
5. 5 M NaOH. Prepare 0.5 M NaOH by mixing 146.1 g of NaOH with 400 mL of water in a beaker containing a magnetic stir bar. Stir until dissolved, and bring volume up to 500 mL with water. Autoclave the solution for 25 min.
6. Tween-20 solution: 0.1 % (v/v) Tween-20. Prepare 50 mL using autoclaved H₂O.
7. Ethyl methanesulfonate (EMS) solution: 0.2 % EMS (e.g., from Sigma-Aldrich, M0880) in autoclaved H₂O (*see* Note 2).

¹Since this protocol is written for modifier screens, use a mutant allele with a weak phenotype to identify enhancers and a mutant allele with a stronger phenotype to identify suppressors. A seed volume of 100 μ L corresponds to ~1,000 seeds.

²EMS is a highly hazardous and volatile compound. Wear double gloves, a lab coat, goggles, and closed-toe shoes when working with this compound. In addition, make sure to prepare all the solutions inside a fume hood. Decontaminate everything that has come in contact with EMS or is touched while working with EMS by washing it with 1 M NaOH and discard waste into the appropriate biohazard container.

8. 0.1 % agar. Add 0.9 g of agar to 90 mL of autoclaved H₂O. Microwave to dissolve the agar and leave to cool at room temperature prior to use (*see* Note 3).

2.1.2 Planting EMS Mutagenized M1, M2, and Mapping Population Seeds—

1. Soil.

2.1.3 Preparing DNA for Map-Based Positional Cloning—

1. Toothpicks and tape for labeling.
2. Mortar and pestle.
3. Liquid nitrogen.

2.1.4 CTAB DNA Extraction—

1. CTAB extraction buffer: 2 % (w/v) cetyltrimethylammonium bromide, 1.4 M NaCl, 20 mM EDTA, 0.2 % (v/v) β-mercaptoethanol, 200 mM Tris–HCl pH 8.0. For 500 mL buffer: 10 g CTAB, 100 mL Tris–HCl pH 8.0 (to make a 500 mL 1 M Tris–HCl stock solution, add 60.55 g Tris base to 400 mL of H₂O, add ~21.1 mL concentrated HCl (pH 8.0), add water to a final volume of 500 mL and autoclave for 25 min), 40.95 g of NaCl, 20 mL of 0.5 M EDTA (to make 500 mL 0.5 M EDTA stock solution, add 84.05 g EDTA to 250 mL H₂O, add 5 M NaOH (~71 mL) slowly while stirring until the EDTA dissolves, pH should be 8, bring up to volume with H₂O and sterilize by autoclaving for 25 min). Prior to use, calculate how much CTAB buffer you will need, transfer that volume to another tube and add 1/500 volume of β-mercaptoethanol (*see* Note 4).
2. Chloroform.
3. Isopropanol.
4. Nanodrop spectrophotometer or any means to quantify the DNA.

2.1.5 Quick and Dirty PCR—

1. Quick and Dirty PCR Extraction Buffer: 200 mM Tris–HCl pH 7.6 (to make a 500 mL 1 M Tris–HCl stock solution, add 60.55 g Tris base to 400 mL of H₂O, add ~28.5 mL concentrated HCl (pH 7.6), add water to final volume of 500 mL and autoclave for 25 min), 250 mM NaCl, 25 mM EDTA, 0.5 % (w/v) SDS (to make a 10 % (w/v) SDS stock solution, add 50 g in 500 mL of distilled water, stir overnight) (*see* Note 5).
2. Small pestles for grinding tissue in 1.5 mL centrifuge tubes.

³Suspending the seeds in 0.1 % agar will allow for better dispersal of the seeds.

⁴All reagents must be added in the order listed. EDTA will not dissolve quickly, so adding the NaOH slowly will allow the EDTA to dissolve when the pH reaches 8.0. Before using the solution, make sure it has cooled down after autoclaving. Make sure to add the β-mercaptoethanol in a hood.

⁵SDS is potentially harmful to the respiratory system. Thus, wear a mask and weigh SDS in the hood.

2.1.6 T-DNA Insertional Mutagenesis: *Agrobacterium*-Mediated Transformation—

1. Flowering *Arabidopsis thaliana* (age is dependent on growth (*see* Note 6).
2. Electrically competent *Agrobacterium tumefaciens* cells.
3. Plant transformation vector containing the T-DNA construct of interest.
4. Plates for plant growth, and antibiotics/herbicides needed for selection of transgenic lines.
5. 5 % (w/v) sucrose solution.
6. Silwet L-77.
7. Trays for floral dipping.
8. Spectrophotometer or other method of measuring cell densities.
9. MS media, LB media, and agar or other growth media.
10. Dark area and/or cover to keep plants away from bright light.
11. Gene Pulser™ (Bio-Rad) or another suitable instrument for *Agrobacterium* transformation, and corresponding electroporation cuvettes.

2.2 Pinpointing the Mutation that Causes the Phenotype

2.2.1 Map-Based Positional Cloning: PCR—

1. 3–4 % agarose gels (*see* Note 7).
2. 10× PCR buffer: 15 mM MgCl₂, 500 mM KCl, 100 mM Tris–HCl (pH 8.3).
3. 2.5 mM dNTPs.
4. Taq DNA polymerase (*see* Note 8).
5. 10× DNA loading buffer: 3.9 mL glycerol, 500 μL 10 % (w/v) SDS, 200 μL 0.5 M EDTA, 0.025 g bromophenol blue, 0.025 g xylene cyanol, bring to 10 mL final volume with distilled H₂O.

2.2.2 TAIL-PCR—

1. Ex-Taq Polymerase kit (Takara).
2. Gel DNA Recovery Kit (Zymo cat#D4001).

2.2.3 Map-Based Cloning via Deep Sequencing—

1. 5 μg RNase-treated, high-quality genomic DNA (*see* Note 9).

⁶It is optional to clip off the first bolts of flowering *Arabidopsis* in order to promote more secondary bolts. Four to six days after clipping, plants can be transformed. Since immature flowers are the organs transformed, plants can be dipped multiple times (2–3 times).

⁷Depending on the size difference between the two bands, 3 % or 4 % gels can be used. The gels can be used up to four times by melting and re-pouring.

⁸Any Taq DNA polymerase should be suitable.

2. Biorupter (Diagenode, #UCD-200 TO).
3. E-Gel® iBase™ Power System (Invitrogen, G6400).
4. E-Gel® 1.2 % General Purpose Agarose 18-Pak (Invitrogen, G501801).
5. E-Gel® 1 kb Plus DNA Ladder (Invitrogen, 10488-090).
6. Agencourt AMPure XP 60 mL kit (Beckman Coulter Genomics, # A63881).
7. TruSeq DNA Sample Preparation kit (Illumina, FC-121-2001).
8. Magnetic stand-96 (Invitrogen, # AM10027).
9. 96-well thermal cycler with heated lid.
10. 96-well 0.3 mL DNase-free PCR plate.
11. Ethanol (200 proof).

3 Methods

3.1

3.1.1 EMS Mutagenesis of Arabidopsis—

1. Place seeds (100 μ L; corresponding to ~1,000 seeds) in a 50 mL conical tube and wash in 30 mL of 0.1 % (v/v) Tween-20 for 15 min at room temperature (RT). Keep the tube shaking constantly (*see* Note 10).
2. Spin down at $400 \times g$ for 1 min at RT.
3. Remove as much solution as possible first using a transfer pipet and then a PIPETMAN.
4. Add up to 15 mL of autoclaved water.
5. Add 30 μ L of EMS to the tube and wrap the cap with Parafilm (*see* Note 11).
6. Mix by inverting the tube several times, and incubate at RT (while shaking) for 8–12 h.
7. Spin the tube at $400 \times g$ for 1 min at RT.
8. Remove the EMS solution, and put it in a separate 50 mL conical tube.
9. Add 5 M NaOH to the tube so that the final concentration is 0.5 M NaOH. Put Parafilm on the tube to seal it and incubate, shaking at RT, overnight. Discard the Parafilm and solution into a biohazardous waste container.
10. Rinse the seeds ten times in 20 mL autoclaved water each (*see* Note 12).

⁹Although the DNA will be fragmented afterwards, DNA integrity is important in order to prepare high quality DNA sequencing libraries.

¹⁰The purpose of using Tween-20 is to allow the EMS to penetrate the seeds more easily. Therefore, this protocol requires less EMS than other protocols.

¹¹Wrapping with Parafilm helps prevent contamination.

¹²Rinsing a copious amount of times helps to get rid of the EMS. Treat the water from the rinse by adding NaOH (to get a 0.5 M final concentration) using the 5 M stock solution.

11. After the last rinse, add 10 mL of autoclaved water and incubate the seeds at RT, while shaking, for 2–4.5 h.
12. Pour off the water, add an additional 10 mL of water to the tube, and using a transfer pipet, transfer water and seeds to a flask containing 90 mL of 0.1 % agar (*see* Note 13).
13. Mix and sow the seeds at 500 μ L per pot with a PIPETMAN using a 1,000 μ L tip.
14. Cover the trays of soil with a lid and leave at 4 °C for 1 week.
15. Transfer the trays to the growth chamber and grow at 23 °C under continuous light.
16. After the plants have developed two leaves, remove the lid (*see* Note 14).

3.1.2 Planting EMS Mutagenized M1, M2, and Mapping Population Seeds—

1. As the M1 plants are growing, check the plants and look for dominant mutations. If a dominant mutant is identified, proceed to **step 10**.
2. Harvest M2 seeds from the individual M1 plants. Place seeds from each plant into a separate tube (*see* Note 15).
3. Poke a small hole in the lid of the tube and leave the seeds to dry for 1 week at RT.
4. Plant the seeds one by one (24–30 seeds per pot), one M2 family for one pot.
5. Place trays at 4 °C for 1 week so the seeds can stratify.
6. Transfer the trays to the growth chamber at 23 °C under continuous light (*see* Note 16).
7. When the plants grow to the two-leaf stage, remove the lid.
8. As the plants flower, assay the plants for the desired floral phenotype (either enhancer or suppressor).
9. Once a mutant phenotype is observed, note the segregation ratio of that phenotype in the pot (*see* Note 17).
10. Backcross the mutant plants to the parental line at least twice before any molecular analysis is performed (*see* Note 18).
11. Cross the mutant plant to another plant (from a different ecotype) to generate the mapping population (*see* Note 19).

¹³The agar helps to suspend the seeds in the solution more evenly, making planting much easier. Remember to allow the 0.1 % agar to cool prior to adding the seeds.

¹⁴The clear, dome-shaped lid maintains moisture to promote germination.

¹⁵Dominant mutations are rare, so most plants will not exhibit an abnormal phenotype. Seeds are collected from individual plants so that mutations in the M2 can be easily maintained in the heterozygous situation if the homozygous plant is sterile.

¹⁶Keeping plants under continuous light allows them to grow faster. Flowering can be accelerated by growing plants under continuous light, which will expedite the genetic screen aimed at the isolation of floral mutants. In addition, the plants should be grown in their own section so as to avoid contamination by other plants in the growth chamber.

¹⁷In the M1 plant, usually only one of two cells that give rise to the germ line gets mutated. The segregation ratio for a recessive mutation in the M2 should be close to 1 out of 8.

¹⁸This is very important as molecular analyses of the mutant should only be performed on a clean line.

12. Generate a “Het” (heterozygous) sample as a control. For example, if the screen was performed in the *Ler* background, cross the mutant plant with another accession (that you will use for mapping, such as Col-0). With the F1 plants, collect the tissue (several leaves) and extract DNA via the CTAB method or the “quick and dirty method” (for Quick and Dirty PCR prep) (*see* Note 20). This sample will be included in subsequent mapping reactions as a control.
13. Using the F2 seeds [from the cross with another accession (same accession that you chose for **step 12**)], plant (24 seeds per pot) the mapping population. Keep in mind that you will, on average, get only 1 out of 4 plants that exhibit the phenotype. For a population of 50 mutant plants, which is needed for a rough mapping of the mutation, at least 200 seeds need to be planted.
14. Place trays at 4 °C for 1 week so the seeds can stratify.
15. Transfer trays to the growth chamber at 23 °C under continuous light.
16. When the plants grow to the two-leaf stage, remove the lid.

3.1.3 Preparing DNA for Map-Based Positional Cloning—

1. Within the F2 mapping population, look for plants with your desired phenotype. If it is a dominant mutation, look for and perform the map-based positional cloning on plants without the desired phenotype (*see* Note 21).
2. Label the plants with toothpicks, and number them. For the rough mapping, 50 plants are needed, which can be pooled (put small samples from many plants in one tube and isolate DNA at the same time). For the pooled method, CTAB DNA extraction is performed for rough mapping. Otherwise, use the Quick and Dirty method on individual plants. For fine mapping, collect tissue from each plant separately, using the Quick and Dirty method.
3. Collect one leaf and place it either in a 15 mL conical tube for the CTAB DNA extraction (in liquid nitrogen) or individually in 1.5 mL centrifuge tubes.
4. Remember to collect tissue from both ecotypes for use as controls (*see* Note 22).

3.1.4 CTAB DNA Extraction—

1. Cool the mortar and pestle in liquid nitrogen.

¹⁹For example, if our mutant were in the *Ler* background, we would cross it to Col-0 to generate the mapping population. Oftentimes, mutants have a stronger or weaker phenotype in the Col-0 background. Thus, there could be an enhancer or suppressor in Col-0, so one could possibly map the modifier. Also, if the mutagenesis is performed in a given mutant background (such as *ag-10*), it is a good idea to introgress the mutation into a different ecotype several times as the mapping parental line (in our case, we crossed *ag-10* to Col-0 six times and used the *ag-10*^{Col-0} as the other parent to generate the mapping population). This is important, as we want to keep two copies of *ag-10* in the subsequent population so that we are able to see the “enhancer” phenotype in a reasonable proportion in the F2 population.

²⁰This DNA is used as a control for future mapping experiments (called Het hereafter). Collect at least four samples for each method because a lot is used for positional cloning.

²¹Positional cloning can also be used to map dominant mutations. In the F2 population, the plants with no mutant phenotypes should be wild type at both alleles, and thus are used for mapping.

²²For simplicity purposes, the controls will be referred to as *Ler* and Col-0. For pooled tissue, make sure the leaves are about the same size f24 or each plant. The tissue can be stored at –80 °C (for the pooled tissue) or 4 °C (for the Quick and Dirty PCR) for several days prior to using.

2. Add frozen tissue to the cold mortar and pestle.
3. Grind the tissue until the powder is light green.
4. Add the tissue to 10 mL of CTAB extraction buffer (with β -mercaptoethanol added) and invert tube several times (*see* Note 23).
5. Place the tubes at 65 °C for 40–60 min. Label two sets of tubes while waiting.
6. Centrifuge for 7 min at $11,648 \times g$ at RT.
7. Transfer the supernatant into a new 15 mL tube.
8. Add an equal volume of chloroform and vortex for 10 s (*see* Note 24).
9. Centrifuge for 15 min at RT at $11,648 \times g$.
10. Transfer the upper phase to a new tube (*see* Note 25).
11. Add an equal volume of cold isopropanol (*see* Note 26).
12. Place the tubes in -80 °C for 30 min or -20 °C overnight (*see* Note 27).
13. Centrifuge tubes for 30 min at 4 °C at $11,648 \times g$.
14. Decant the supernatant into a liquid waste container.
15. Wash pellet twice with 4 mL of 70 % EtOH (*see* Note 28).
16. Invert the tube and leave the tube in the hood to air-dry (*see* Note 29).
17. Once the pellet is dry, add 100 μ L of autoclaved water for resuspension (*see* Note 30).
18. If you wish to remove RNA, add RNase A to a final concentration of 20 μ g/mL and incubate at 37 °C for 1 h. If not, continue with **step 29**.
19. Add water to a final volume of 300 μ L.
20. Add 150 μ L phenol (high pH) and 150 μ L chloroform.
21. Briefly vortex.
22. Spin in a tabletop microfuge at $16,100 \times g$ for 15 min.
23. Transfer supernatant to a new, clean tube and add an equal volume of isopropanol.

²³The mixture should be dark green and viscous but not too thick. If it is too thick, the solution is hard to move when inverted. In this case, split the samples into different tubes and add more extraction buffer. If CTAB extraction is being performed on one plant (several leaves), no liquid nitrogen is needed. Use a blue pestle (for microcentrifuge tubes) and grind the tissue in a 1.5 mL centrifuge tube. All spins can be done in a tabletop microfuge at $16,100 \times g$.

²⁴The solution should be cloudy.

²⁵Be careful not to jostle the tube to disrupt the separation. If the tube is jostled, re-spin. This phase should be clear.

²⁶Be careful not to transfer any chloroform at this step.

²⁷Theoretically, the DNA can be saved at -20 °C indefinitely as it is stable; however, it is best to use the DNA as soon as possible.

²⁸For the first wash, add 4 mL of 70 % EtOH very slowly and invert carefully so as to avoid dislodging the pellet. If the pellet is dislodged, spin for 5 min and wash again. If you do not dislodge the pellet, decant 70 % EtOH, add more 70 % EtOH, and invert the tube vigorously. Spin down the tube at $11,648 \times g$, 4 °C for 5 min.

²⁹Since the pellet may disappear after drying, make a mark on the outside of the tube to show the pellet's location. That way, when the pellet is resuspended, autoclaved water can be added up to that point.

³⁰Use a tip to help resuspend the pellet. Warming up the water (37 °C) also helps with the resuspension. If there was a small amount of tissue used, resuspend in 30 μ L of autoclaved water.

24. Leave at $-80\text{ }^{\circ}\text{C}$ for 30 min (*see* Note 31).
25. Spin in a tabletop microfuge at $16,100 \times g$ for 25 min at $4\text{ }^{\circ}\text{C}$.
26. Wash twice with 70 % EtOH, as in **step 15**.
27. Invert and leave the tube to air-dry in the hood.
28. Resuspend in 100 μL autoclaved water, as in **step 17**.
29. Quantify the DNA.
30. Store DNA at $-20\text{ }^{\circ}\text{C}$ (*see* Note 32).

3.1.5 Quick and Dirty Extraction—

1. Grind tissue in an 1.5 mL Eppendorf tube (*see* Note 33).
2. Add 400 μL Quick and Dirty extraction buffer to the tube.
3. Grind a little more until you do not see chunks of tissue.
4. Spin in a tabletop microfuge at $16,100 \times g$ for 5 min at RT.
5. Transfer the supernatant to a new tube (*see* Note 34).
6. Add an equal volume of isopropanol.
7. Leave the tubes at RT for 15 min (*see* Note 35).
8. Spin down the samples in a tabletop microfuge for 10 min at RT at $16,100 \times g$.
9. Decant the supernatant.
10. Wash pellet twice with 150 μL 70 % EtOH.
11. For the last wash, spin the tubes in a tabletop microfuge for 5 min at RT at $16,100 \times g$.
12. Decant the supernatant and leave the inverted tubes/plates to dry in the hood.
13. Add 50 μL of autoclaved water to the pellet.
14. Leave DNA at RT for 1 h if performing the PCR on the same day. Use 1 μL for a PCR reaction with a 10 μL final volume.
15. Store the DNA at $-20\text{ }^{\circ}\text{C}$.

³¹The solution can also be left at $-20\text{ }^{\circ}\text{C}$ for a few hours to overnight. If left overnight, add a 1/10 volume 3 M sodium acetate and 1 μL glycogen, as a carrier, to the solution.

³²If the DNA is used the same day, leave the tubes at room temperature for 1 h.

³³Keep elbow above the wrist when grinding. This will allow better grinding without using that much energy and/or causing wrist injury. When performing extraction for hundreds of plants, this helps.

³⁴If hundreds of preps are being performed, it might be easier to use the plate method. In this case, add 140 μL of the supernatant to each well of a 96-well PCR plate, which contain 140 μL of isopropanol. Because this leads to the well being almost completely full, be careful to avoid cross-contaminations between individual wells. As a precaution and to allow you to repeat the experiment in case of a contamination, keep the tubes with the leftover supernatant.

³⁵Do not leave the tubes in the freezer or at $4\text{ }^{\circ}\text{C}$ as this will result in DNA preparations with a high level of impurities.

3.1.6 T-DNA Insertional Mutagenesis: Transformation and Selection of *Agrobacterium*—

1. Add 0.5 mL (2–10 ng) plasmid to 50 mL of electrically competent *Agrobacterium* cells on ice (*see* Note 36).
2. Cool a 0.2 cm electroporation cuvette (Bio-Rad) on ice.
3. Add the entire plasmid and competent cell mixture to the cuvette.
4. Place the electroporation cuvette in the Gene Pulser™ (Bio-Rad). Make sure to select the setting for bacteria, and press the pulse button. Remove the cuvette from the machine and place it on ice.
5. Pipette the mixture carefully out of the cuvette and into a 1.5 mL tube containing media (LB or other). This should be done in the flow hood to help reduce contamination.
6. Shake for 1–3 h in a 28 °C shaker.
7. Pipette the mixture carefully out of the tube and onto a selection plate containing the appropriate media (LB or other) and antibiotic. Make sure the mixture is spread evenly on the surface of the plate. Let it air-dry in the flow hood.
8. Incubate this plate overnight at 28 °C or until colonies form (*see* Note 37).
9. Prepare a test tube of 5 mL media of choice and antibiotic. Inoculate a colony into the liquid media using a pipet tip by touching the colony. Then, dip the tip end into the media, pipetting if desired. The tip can also be placed into the media if necessary.
10. Incubate the 5 mL tube on a 28 °C shaker overnight.
11. The presence of the desired DNA construct can be verified by colony PCR (*see* Note 38).
12. Store *Agrobacterium* if needed (*see* Note 39).

3.1.7 T-DNA Insertional Mutagenesis: Preparation of *Agrobacterium*—

1. The next morning, pour the 5 mL *Agrobacterium* culture into 500 mL (1:100) LB, or other growth media. Make sure to add the antibiotics for selection. Grow at 28 °C until OD₆₀₀ = 0.8 (*see* Note 40).

³⁶There is an optional step of keeping the mixture of competent cells and plasmid on ice 30 min prior to electroporation.

³⁷Colonies should form within a couple days. If they do not form, the transformation was not successful and should be repeated. These plates can be stored at 4 °C for a few months at a time.

³⁸To make sure a positive colony is obtained, multiple colonies can be inoculated into test tubes (5 mL media with antibiotics). Multiple colonies can also be verified using colony PCR to save time.

³⁹To store bacteria, inoculate another 5 mL media and antibiotics in a test tube with a positive colony. Grow overnight in a 28 °C shaker. Pipette 150 µL sterile glycerol into a 1.5 mL plastic tube. Pipette 850 µL bacterial culture into the same tube and mix well. It will take some effort to mix the two completely. Label the tube and flash freeze it in liquid nitrogen. Store in –80 °C until needed for future transformations.

⁴⁰This OD₆₀₀ requirement is not strict. Generally, 0.6–0.8 is used. However, as long as one can suspend the *Agrobacterium* to about an OD₆₀₀ = 0.8 in a solution of 5 % sucrose, then efficiency of transformation will not be adversely effected. The time will be about 18–24 h of incubation. Alternatively, the *Agrobacterium* can be grown to 1.0–1.2 and diluted to 0.8.

2. Spin down the *Agrobacterium* ($5,500 \times g$, 15 min at RT) and resuspend in a 5 % sucrose, $0.5 \times$ MS solution, pH 5.7. For example, if the $OD_{600} = 0.8$ in 500 mL LB, the OD_{600} will also be 0.8 in 500 mL of 5 % sucrose solution. However, only about 200–300 mL of solution is needed for dipping of 1–3 trays of *Arabidopsis* (*see* Note 41).
3. Add 0.05 % (v/v) of Silwet L-77 to the solution and mix well (*see* Note 42).
4. Pour the solution into a low, flat tray. This solution does not need to be autoclaved if used quickly.

3.1.8 T-DNA Insertional Mutagenesis: Floral Dip—

1. Dip the shoots of plants into the solution, and let sit for 5 min. Do not let the soil touch the solution (*see* Note 43).
2. If transforming *Ler* plants, you must put the solution and pot (from **step 1**) into a vacuum for 5 min. Release the vacuum slowly after 5 min. This step can be skipped for Col-0 plants, which are easier to transform.
3. Spray the shoots of the plants with water, using a spray bottle, for several seconds to remove the Sucrose solution.
4. Cover the plants with a plastic dome or other covering for about 24 h. Plants are usually stored on their sides during this time.
5. Return plants to the upright position and grow them until they are ready for seed harvesting.
6. Collect seeds from the plants and seeds from many plants can be pooled.

3.1.9 T-DNA Insertional Mutagenesis: Selection of Transformants—

1. Grow the seeds on soil and spray with herbicide such as ammonium-glufosinate (“BASTA”) to select transformants. Remember to stratify the seeds for 2–5 days after planting.
2. To select transformants with an antibiotic, prepare plates of $0.5 \times$ MS and 0.6 % agar with the antibiotic added in the correct amount. Grow under continuous light for 7–10 days.
3. Positive transformants will appear larger than seedlings that were not transformed.
4. Transfer transformants to soil, and collect seeds from either a single plant or a few plants (5–50).

3.2

3.2.1 Map-Based Positional Cloning: PCR—

⁴¹Each tray is sized $21'' \times 11'' \times 2''$ with 12 pots, in which multiple plants of the same genotype are grown.

⁴²Standard is 0.05 % (v/v) Silwet L-77, but as low as 0.005 % (v/v) can be used successfully. Less should only be used when toxicity is a risk to plants.

⁴³If the solution touches the soil, the high sugar concentration will change soil pH, leading to unhealthy plants.

1. The PCR reaction mix is as follows for a 10 μ L reaction: 1 μ L of Quick and Dirty DNA (or 50 ng of CTAB DNA), 0.1 μ L each of 10 mM forward and reverse primer, 0.4 μ L of 2.5 mM dNTPs, 1 μ L 10 \times PCR buffer (contains 18 mM MgCl₂), 0.1 μ L of Taq DNA polymerase, and 7.3 μ L of autoclaved water (*see* Note 44).
2. The PCR reaction conditions are as follows: step 1: 94 $^{\circ}$ C for 3 min; step 2: 94 $^{\circ}$ C for 30 s; step 3: 54 $^{\circ}$ C for 30 s; step 4: 72 $^{\circ}$ C for 30 s; repeat steps 2–4 for an additional 34 times; step 5: 72 $^{\circ}$ C for 10 min.
3. Run the PCR product on a 3 % or 4 % agarose gel. Make sure to add EtBr before pouring the gel since EtBr is not easily absorbed into a higher percentage gel after the gel is made. Be careful when handling EtBr, it is a mutagen. The lanes should be set up as follows: pooled DNA from mutant plants; *Ler*; Col-0; DNA from F1 plants or *Ler* and Col DNA mixed in a 1:1 ratio.

3.2.2 Map-Based Positional Cloning: Analysis—

1. For the rough mapping via the pooled method, if the mutant is in the *Ler* background, one should look for the set of primers that show a predominant *Ler* pattern (*see* Note 45).
2. Once the region that is linked to the mutation is identified, fine mapping can be started.
3. Isolate the DNA individually for each mutant (as described above).
4. Go to <http://www.arabidopsis.org>. Click “sequence viewer” and identify BACs flanking the region in which the mutation is linked.
5. Find or design SSLP, CAPS, or dCAPS markers in the BAC. One useful resource is <http://amp.genomics.org.cn/>, which lists such markers. Alternatively, use the SNP information from <http://signal.salk.edu/atg1001/3.0/gebrowser.php>. Design primers that flank the SNPs (*see* Note 46).
6. Run a PCR reaction and resolve the product on an agarose gel.
7. For the analysis, find the region flanked by recombinant markers. An example is shown in Table 3. Assume *ciw8* was the marker to which the mutant was linked. Markers 1–5 are in the vicinity of the linked region. Results from plant 1 show that the mutation is below Marker 1. Results from plant 2 tell us that the mutation is between Marker 2 and Marker 5. Results from plant 3 tell us that the mutation is between Marker 1 and 4. Results from plant 4 tell us that the mutation is between Marker 3 and 4. In summary, the mutation is between Marker 3 and Marker 4. The mutation is localized to the region flanked by two markers, one on each side, with the least number of recombinants in the population.

⁴⁴Make sure to run both ecotypes and 1:1 mix of the two accessions as controls for all reactions.

⁴⁵If the mutant is in the Col-0 background, identify the marker that is linked to Col-0.

⁴⁶Go to the markers tab and insert BAC number. A set of primers will be displayed. The most commonly used polymorphism information between Col-0 and *Ler* is <http://www.arabidopsis.org/browse/Cereon/index.jsp>; however, the one mentioned in the actual protocol is more comprehensive.

8. Once a region of 200 kb or less is identified, go to <http://www.arabidopsis.org>, find the region in Seqviewer, and scan the region to see if there are any genes of interest. If so, sequence the gene from the mutant to find any mutations. To narrow down the region further, more plants will be needed for fine mapping.
9. If a mutation is identified, complementation analysis should be performed.

3.2.3 TAIL-PCR—The TAIL-PCR is comprised of three separate PCR reactions as outlined below.

1. The first reaction is the pre-amplification reaction. *see* Table 4 for the set-up (*see* Note 47).
2. The PCR conditions for the first reaction are shown in Table 5.
3. The second reaction is the Primary TAIL-PCR reaction. *see* Table 6 for the set-up (*see* Note 48).
4. The PCR condition for the second reaction is shown in Table 7.
5. The third reaction is the Secondary TAIL-PCR reaction. *see* Table 8 for the set-up (*see* Note 49).
6. The PCR condition for the third reaction is shown in Table 9.
7. Run the Primary and Secondary TAIL-PCR reactions on a 1.5 % agarose gel.
8. Choose the PCR product with clean bands and a proper ladder effect (*see* Note 50).
9. Weigh an empty tube, and record the weight (*see* Note 51).
10. Take an image of the gel (*see* Note 52).
11. Put the gel on top of plastic wrap, and place it on top of a UV light source (*see* Note 53).
12. Cut out the band (*see* Note 54).
13. Place the band into the pre-weighed 1.5 mL centrifuge tube.
14. Weigh the tube, and subtract the weight of the tube without the gel (*see* Note 55).
15. Multiply the weight of the agarose by three. Add that volume (in μL) of ADB buffer (*see* Note 56).
16. Leave the tube at 37–55 °C for 5–10 min (*see* Note 57).

⁴⁷There will be a total of four different PCR tubes for this reaction (one for each one of the LAD primers).

⁴⁸Once again, there will be a total of four different PCR tubes for this reaction (one for each one of the LAD primers).

⁴⁹Once again, there will be a total of four different PCR tubes for this reaction (one for each one of the LAD primers).

⁵⁰The band from the Secondary PCR reaction should run faster than the band from the Primary PCR reaction. As the product will be used for sequencing, make sure everything is clean. For instance, clean the gel apparatus, use new running buffer, etc.

⁵¹Use a balance that measures the weight to the precision of at least 1 mg.

⁵²Do this as fast as possible as long exposure to the UV light will induce thymine dimers that affect subsequent PCR reactions.

⁵³Make sure to wear a UV mask to protect eyes and skin from burns.

⁵⁴Make sure to use a new razor blade and get rid of as much agarose as possible.

⁵⁵This is to find the weight of the gel containing the band.

⁵⁶For example, if the weight of the agarose band is 100 mg, add 300 μL of ADB buffer.

17. Transfer the solution containing the melted gel to a Zymo-Spin™ Column in the collection tube.
18. Leave the solution in the column for 3 min.
19. Spin the column in a tabletop microfuge for 30 s at RT at $16,100 \times g$.
20. Discard the flow-through.
21. Add 200 μL of wash buffer to the column (*see* Note 58). 22. Let the column sit at RT for 3 min.
22. Centrifuge for 30 s at $16,100 \times g$ in a tabletop microfuge. Discard the flow-through.
23. Repeat the wash step.
24. Centrifuge the empty column for 1 min at $16,100 \times g$ in a tabletop microfuge.
25. Transfer the column to a new, labeled centrifuge tube.
26. Add 25 μL water to the column (*see* Note 59).
27. Let the column sit at RT for 3 min.
28. Centrifuge the column for 2 min at $16,100 \times g$ in a tabletop microfuge.
29. Quantify the DNA, and run $\sim 2\text{--}5 \mu\text{L}$ of DNA on the agarose gel to make sure there is a clean, single band.
30. Send the required amount to the sequencing facility per their specifications.

3.2.4 Map-Based Cloning via Deep Sequencing: Library Preparation—

1. Obtain 5 μg RNase-treated genomic DNA via the CTAB method above with the exception of resuspending in 50 μL autoclaved water at the final step.
2. Use the bioruptor to sonicate the DNA. Perform two replicates of 15-min sonication with 30 s on at maximum speed and 30 s off (*see* Note 60).
3. Recycle the DNA between 200 and 300 bp through the E-gel system (*see* Note 61).
4. Build a DNA sequencing library using the TruSeq DNA Sample Preparation kit.
5. Sequence the library using the Illumina Genome Analyzer (*see* Note 62).

⁵⁷Every few minutes, briefly vortex the tube to help the agarose dissolve. Running the PCR product on a higher percentage gel will cause the gel to be harder to dissolve. It may take longer than 10 min for the gel to fully dissolve.

⁵⁸Make sure to add the required amount of ethanol to the wash buffer prior to using.

⁵⁹Be careful not to let the tip touch the column.

⁶⁰The genomic DNA can be fragmented by any kind of sonication method as long as the DNA is sheared to 200–300 bp. Run a 1 % agarose gel to check. A smear is expected, but the majority of the band intensity should be between 200 and 300 bp.

⁶¹This step can be skipped if the gel method is used to purify the ligation product before enrichment of the DNA fragment when preparing the DNA sequencing libraries. Also, this can be done through regular gel purification methods (as mentioned above with the Zymo kit). If purifying the DNA at this step, there is no need to purify the ligated DNA during the next part of the procedure.

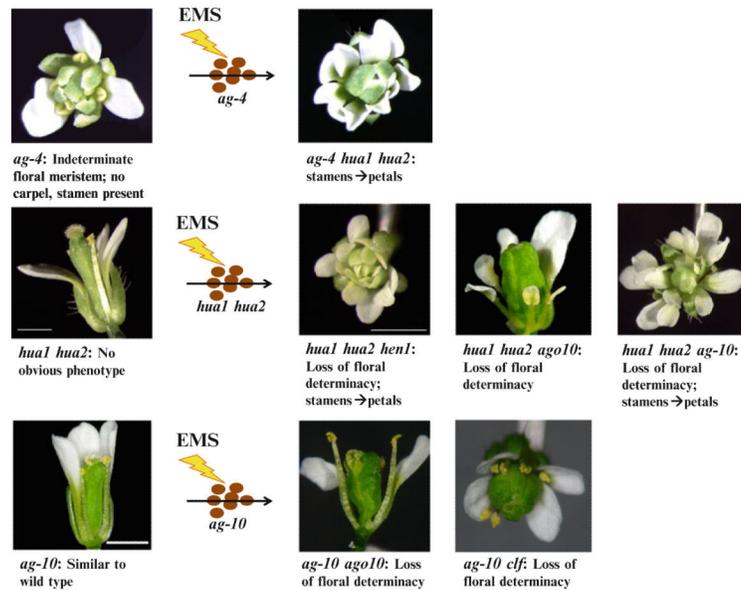
⁶²Multiple samples can be barcoded in a single lane to lower costs of sequencing. However, the coverage for the genome should be higher than 10.

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**Fig. 1.**

EMS Mutagenesis is a Powerful Tool to Dissect Pathways Governing Floral Development. An outline of several EMS-based enhancer screens used to dissect the floral determinacy pathway governed by *AG*. A relatively weak allele of *AG*, *ag-4*, is defective in carpel identity and floral determinacy; however, stamens are still present. EMS mutagenesis was performed on *ag-4* seeds, resulting in the identification of the genes *HUA1* and *HUA2*, mutations in which convert the stamens in *ag-4* to petals. The *hua1 hua2* mutant has weak organ identity defects but is normal in floral determinacy. Another EMS mutagenesis experiment was performed on *hua1 hua2* and another *ag* allele was isolated (*ag-10*), as well as mutations in *HEN1* and *AGO10*. *ag-10* has a very weak phenotype, only a few siliques in a plant are slightly bulged, indicative of loss of determinacy. Another EMS mutagenesis experiment was performed on *ag-10*, and mutants in genes such as *CLF* and *AGO10* were isolated. Thus, *CLF* and *AGO10* are involved in floral determinacy

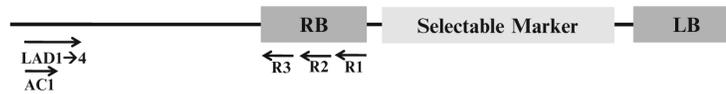


Fig. 2.

Schematic diagram depicting the identification of a T-DNA insertion site. A basic schematic of the T-DNA insertion region and location of all primers used to identify the site of integration is shown. The *dark grey boxes* represent the right and left border (RB and LB, respectively). The *light grey portion* represents the region where the selectable marker can be placed. The T-DNA region (the region that will be integrated into the host genome) encompasses everything from the LB to RB (with the selectable marker). The LAD1→4 primers are each used with R1 in the four initial Tail-PCR reactions. AC1 in conjunction with R2 and R3 primers are used in subsequent nested PCR reactions

Table 1

Oligonucleotide sequences for map-based positional cloning

	Primer name	Sequence (5'→3')	Primer name	Sequence (5'→3')	Position
CHR1	F21M12-F	GGCTTTCTCGAAATCTGTCC	F21M12-R	TTACTTTTGGCCTCTGTGTCATTG	AT1G09880
	1CER458676F	CGTTTGAAACACACTACAGGATTAC	1CER458676R	AGCTCAAACCTTAAGCATAGAAAACC	AT1G27520
	F28H19-F	TGCGGGGAGTGTGATAGAAATA	F28H19-R	TCCTCGAAAAGATTTCATTGAT	AT1G43763
	F2401-F	TCACAAAAAATGCAACATTTA	F2401-R	TAATGGCTCCAATCAATA	AT1G62370
CHR2	1CER470018F	GATCATAATTCTATTGACCCCATCAG	1CER470018R	CTTACGGGTTTACTGTCTCATGTTT	AT1G71930
	T23K3-F	CGTGTTTACCGGGTCCGA	T23K3-R	AAAAACCCTTGAAGAAATACG	AT2G01860
	2CER449854F	GACGACTTCGAGAAAGTTACAAAAAC	2CER449854R	AACGGAGTAATAACTCCAATCTCATC	AT2G13851
	T20P8-F	TCCGATTCCGATTAACACTC	T20P8-R	TTAATTCCTAATTTCAAGACT	AT2G27130
CHR3	T3D7-F	GGTATCGATTGAGCAAAATAA	T3D7-R	ACATGCGTCTGCTTGGAG	AT2G47160
	F24P17	ACTGCACATTGCACGAAACA	F24P17	GGATGGCAACTTAGGCTGAA	AT3G06400
	K1G2-F	ATGAGCTTTAGGAGTGTGTA	K1G2-R	AATTTGTCCCAAAAAGAATA	AT3G27460
	CIW4-F	GTTCATTAACACTTGCCTGTGT	CIW4-R	TACGGTCAAGATTGAGTGAITTC	AT3G50820
CHR4	T20010-F	AAATGCCAGGGGAATAGA	T20010-R	CAAAACCATGCAATGATGC	AT3G62988
	CIW5-F	GGTTAAAAAATTAGGGTTACGA	CIW5-R	AGATTTACGTGGAAAGCAAT	AT4G01710
	CIW6-F	CTCGTAGTGCACTTTCATCA	CIW6-R	CACATGGTTAAGGAAAACAATA	AT4G13575
	4CER450255F	CACAAAGACAACACCAAAAAAC	4CER450255R	AGAAAGGAATGGCTTCATCTA	AT4G20095
CHR5	AP22-F	ATTATGTTAAGGAAAAATGAGAT	AP22-R	GGGTTGTAAGAAATTAAGAA	AT4G36780
	T31P16-F	TCGAAAGTAACCTTACTTTCTA	T31P16-R	AATGTCGCAAAAGACTTCC	AT5G10040
	CIW8-F	TAGTGAACCTTTCTCAGAT	CIW8-R	TTATGTTTTTCTTCAATCAGTT	AT5G22550
	ATPHYC-F	CTCAGAGAAATCCAGAAAAATCT	ATPHYC-R	AAACTCGAGAGTTTTGTCTAGATC	AT5G35840
MRB17-60F	MJB21-38F	CGATGCTCAGGTTCTACATT	MJB21-38R	ACTAAAAATATCATCTCTCGTTTGTGA	AT5G42720
	MRB17-60F	CGAGCAAAATGAATCTGAAGG	MRB17-60R	GTGATAAATTCGTAATAATATGGACT	AT5G54630

Table 2

Oligonucleotide sequences for TAIL-PCR

Name	Sequence (5'→3')
LAD1	ACGATGGACTCCAGAGCGGCCGC(G/C/A)N(G/C/A)NNNGGAA
LAD2	ACGATGGACTCCAGAGCGGCCGC(G/C/T)N(G/C/T)NNNGGTT
LAD3	ACGATGGACTCCAGAGCGGCCGC(G/C/A)(G/C/A)N(G/C/A)NNNCCAA
LAD4	ACGATGGACTCCAGAGCGGCCGC(G/C/T)(G/A/T)N(G/C/T)NNNCGGT
AC1	ACGATGGACTCCAGAG
R1	cct cta gag tgc acc tgc agg cat g
R2	acg atg gac tcc agt ccg gcc aac tta
R3	tga atg gcg aat gct aga gca gct t

Table 3

Example of analysis of F2 mapping population

	Marker 1	Marker 2	Marker 3	Ciw8	Marker 4	Marker 5
Plant 1	Het	<i>Ler</i>	<i>Ler</i>	<i>Ler</i>	<i>Ler</i>	<i>Ler</i>
Plant 2	Het	Het	<i>Ler</i>	<i>Ler</i>	<i>Ler</i>	Het
Plant 3	Het	<i>Ler</i>	<i>Ler</i>	<i>Ler</i>	Het	Het
Plant 4	Het	Het	Het	<i>Ler</i>	Het	<i>Ler</i>

Table 4

TAIL-PCR set-up for the pre-amplification reaction

Reagent	Final concentration	Amount to add (μL)
H ₂ O		12.7
10 \times PCR buffer		2
dNTP	200 μM	1.6
LAD 1–4 primer	1 μM (10 μM stock)	2
R1 primer	0.3 μM (10 μM stock)	0.6
ExTaq	0.5 units	0.1
DNA template	50 ng	1
Total		20

Table 5

TAIL-PCR conditions for the pre-amplification reaction

Step	Temperature (°C)	Time (min:sec)
1	93	2:00
2	95	1:00
3	94	0:30
4	60	1:00
5	72	3:00
6	Go to step 3	10 times
7	94	0:30
8	25	2:00
9	Ramping to 72	0.5 °C/s
10	72	3:00
11	94	0:20
12	58	1:00
13	72	3:00
14	Go to step 11	25 times
15	72	5:00
16	End	

Table 6

TAIL-PCR set-up for the primary reaction

Reagent	Final concentration	Amount to add (μ L)
H ₂ O		17.88
10 \times PCR buffer		2.5
dNTP	200 μ M	2
AC1 primer	0.3 μ M (10 μ M stock)	0.75
R2 primer	0.3 μ M (10 μ M stock)	0.75
ExTaq	0.6 units	0.12
DNA template	1/40 dilution of the pre-amplification product	1
Total		25

Table 7

TAIL-PCR conditions for the primary reaction

Step	Temperature (°C)	Time (min:sec)
1	94	0:20
2	65	1:00
3	72	3:00
4	Go to step 1	1 time
5	94	0:20
6	68	1:00
7	72	3:00
8	94	0:20
9	68	1:00
10	72	3:00
11	94	0:20
12	50	1:00
13	72	3:00
14	Go to step 5	13 times
15	72	5:00
16	End	

Table 8

TAIL-PCR set-up for the secondary reaction

Reagent	Final concentration	Amount to add (μL)
H ₂ O		35.8
10 \times PCR buffer		5
dNTP	200 μM	4
AC1 primer	0.3 μM (10 μM stock)	1.5
R3 primer	0.3 μM (10 μM stock)	1.5
ExTaq	1 unit	0.2
DNA template	1/10 dilution of the primary reaction PCR product	1
Total		50

Table 9

TAIL-PCR conditions for the secondary reaction

Step	Temperature (°C)	Time (min:sec)
1	94	0:20
2	68	1:00
3	72	3:00
4	94	0:20
5	68	1:00
6	72	3:00
7	94	0:20
8	50	1:00
9	72	3:00
10	Go to step 1	7 times
11	72	5:00
12	End	