Chapter 1
Classical mutagenesis in higher plants

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1 Introduction

Mutagenesis aims at the disruption or alteration of genes. Mutants are the basis of genetic variation and therefore have attracted the interest of plant breeders. For a long time, mutagenesis research in plants focused on crop improvement and, especially for crop plants, optimized protocols were developed with barley being one of the favourite species. However, the interest in mutagenesis has shifted to basic plant research in the last 20 years, when the power of mutant approaches in combination with molecular techniques to investigate the molecular nature of the genes became fully appreciated.

Mutations can be induced by chemical, physical, and biological means. The latter technique, where genes are disrupted by insertion of DNA, originating from primary transformation events (see chapter 2 and 3) or from transposable elements (see chapter 4), became very popular because of the subsequent ease to clone the mutated gene. Although the application of the biological mutagens differs from the use of the 'classical' chemical and physical mutagens, which are the topic of this chapter, many of the basic principles are the same.

The present outline on mutagenesis procedures is based on experience with self-fertilizing species, such as the model plants *Arabidopsis thaliana* and tomato. The basic principles of mutagenesis in these species also hold for other self-fertilizing species. Several reviews and detailed protocols for classical mutagenesis of *Arabidopsis* have been published (1–3).

Differences in mutagenesis procedures between self-fertilizing species, cross-fertilizing species, and plants that are vegetatively propagated, are that, for the latter two types of species, the parental plants are often heterozygous at many loci. As a consequence, mutations in dominant alleles at these loci are recognizable as mutants in the mutagen-treated plants. In the case of vegetatively propagated plants, plant parts instead of seeds are treated. Because mutations are induced in single cells, mutations in such multi-cellular structures will appear as mutant sectors (chimeras) deriving from the cell that contains the mutation. Non-chimeric mutants can be obtained by propagating the mutant

sectors vegetatively. This can be done with classical techniques or *in vitro* culture. For self-fertilizing species, dominant mutations are visible in the plants derived from mutagenized seed, but recessive mutations can only be identified in their progeny. A handbook describing many details of mutagenesis of such plants and crops has been published by van Harten (4).

Cross-pollinators that can be selfed, such as maize, *Brassica* sp., etc., can be treated in mutagenesis experiments as self-pollinators, following similar procedures as described in this Chapter, although generating selfed progenies will often be more laborious. When selfing is not possible, one will need other inbreeding procedures such as sib-mating to reveal the presence of homozygous recessive mutants in the progeny of mutagen-treated plants.

Tetraploid and allotetraploid plants are not very useful for mutagenesis experiments because they contain four copies of every gene in their genomes. This implies that genotypes that are homozygous for the recessive mutant allele will not appear in the selfed progeny when the parental genotype contained four wild-type alleles.

2 General principles and nomenclature

Most mutations lead to a loss of function of a functional gene. Genetically, such mutations behave as recessive, which implies that the mutant phenotype is not observed in the plant in which the mutations occurred. This generation of plants grown from the mutagen-treated parental genotype is called the M1 generation and is heterozygous for mutations. The embryos within the seeds formed on such plants and the plants that grow out of these seeds represent the M2 generation, in which homozygous recessive mutations will segregate. It is relevant to realize that the outer layer of seeds (testa) is of maternal origin. This means that mutants defective in the seed coat (of which many seed colour mutants are examples) can only be detected among the seeds developing on the M2 plants, which themselves have M3 embryos.

The material to be treated with mutagen is usually seed, which are multicellular structures. Since the genome is damaged randomly in each cell, different cells of the same seed will contain different mutations. M1 plants, derived from the mutagenesis of seeds, will therefore be chimeric. For the detection of mutations only those that occurred in cells that form the germline can be detected in the M2 generation. The number of cells that contribute to the germline was called the genetically effective cell number (GECN) by Li and Rédei (5), and estimated to be, on average, two in *Arabidopsis*. The consequence of this is that half of the progeny of an individual M1 plant derives from a sector in which a specific gene (*A*) is not mutated (*AA*), and the other half from the sector that was heterozygous for this gene (*Aa*). The segregation ratio observed for *aa* in the progeny of this chimeric plant is then 7 *A* (*AA* + *Aa*) : 1 *aa*. This 7 : 1 ratio is obtained from a 4 : 0 ratio from the non-mutated sector and a 3 : 1 ratio from the sector carrying the mutated allele *a*. The sectors that originate from the germline cells present in seeds are not identical in size and their contribution to the

main inflorescence on which seeds are harvested changes during development. Parts of the plant that are formed later (the top) are mostly composed of only one sector derived from only one germline cell. The consequence of this is that variation occurs between M1 plants and that the harvesting policy also determines how many sectors contribute to the progeny. Chimerism disappears when the mutation is transmitted through the progeny because every individual of this progeny is derived from a single zygote cell.

In monoecious plants, such as maize, where male and female flowers are separated and almost always derive from different germline cells, the selfed progeny of a mutagen-treated seed will not segregate for the recessive mutation because a recessive allele is only provided by one of the gametes. Another generation of selfing will reveal the recessive mutants (in the M3). To bypass this chimerism problem, maize geneticists generally treat pollen with the mutagens, which they apply to a pistillate parent on which the M1 seeds are thereafter harvested (6). Each M1 plant will then be non-chimeric and recessive mutants will appear in the selfed progeny (M2 generation). Protocols for EMS mutagenesis of maize pollen and seeds have been published by Neuffer *et al.* (6).

Chimerism should also be taken into account when transposable elements are used as mutagens (see chapter 4). In this case, sector size is much more variable and depends on when during development the insertion occurred. In the case of transposable elements, chimerism is reintroduced when the element moves again.

Mutation frequencies (m) should be based on the frequency at which mutations occur per treated cell. Usually, these are expressed per locus or per group of mutants.

In practice, these estimates are based on the estimate of the number of mutants found in the M2 in relation to the number of M2 plants that are screened. It is also possible to base the estimate on the number of M1 plants whose progeny were screened for mutants.

The method of Gaul (see 7, 8, and references therein) is most simple and expresses the number of mutants found per number of M2 plants divided by f, which is the average mutant frequency in the progeny of a heterozygote (f = 0.25 for recessive mutants).

Therefore $m = m'/n \times f$ in which m' is the number of mutants found per n M2 plants screened. This estimate is independent of the number of M1 plants from which these are derived and also from the degree of chimerism. By dividing m by 2, the frequency of mutation per haploid genome can be obtained. Li and Rédei (5) derived the same frequency from the number of M1 plants whose progeny were tested. This procedure takes into account the GECN, and also requires that all mutations will be detected and that, therefore, the size of the progeny of individual M1 progenies should be large enough not to miss the mutant by chance. When GECN = 2, the mutant occurs only at a frequency of 1/8 which requires 23 plants not to miss the *aa* plant by chance in a given progeny at $P < 0.05$.

The mutation frequency per cell is calculated according to the method of Li and Rédei (5) with the formula: $m = M/(S \times \text{GECN})$, where M is the number

of M1 progeny segregating for a mutation (type) among S progeny that were screened.

Estimates for a number of loci and mutant groups are given for EMS and irradiation by different authors (1, 3, 5, 7) to be between 10^{-5} and 10^{-3} per locus per haploid genome for effective treatments. This implies that mutants with lesions at specific loci can be found with a reasonable chance in the progeny of a few thousand M1 plants (see discussion on numbers hereafter). These frequencies are lower when only specific base pair changes give a certain, often dominant, phenotype.

Although some literature has suggested that the mutation frequency per locus depends on the genome size, data provide no evidence for this (7).

3 Choices in a mutagenesis experiment

Important decisions to be made in a mutagenesis experiment are the choice of the parental genotype, the type of mutagen, the dose of mutagen, the number of M1 plants to be grown, and the number of M2 plants to be screened.

3.1 Parental genotypes

The choice of parental genotype determines which mutations can be found. An obvious, but often neglected explanation for this is that so-called wild type plants can be mutant for specific loci (reviewed in 9). In *Arabidopsis*, the frequently used line Landsberg *erecta* (L*er*) behaves as a mutant of the flowering inhibitor gene *FLC*. The latter gene is epistatic to several other flowering time genes, which implies that mutants in such genes are not expressed in the L*er* background. Another standard line Wassilewskija (Ws1) is mutant for the phytochrome D (*PHYD*) and the cauliflower (*CAL*) gene. The latter mutation is not observed because it is only expressed in an *ap1* mutant background, which indicates that *AP1* and *CAL* have a similar function. This gene redundancy, for which genotypes can differ, provides another reason for differences in the possibility to find specific mutants in different genetic backgrounds. The various wild types often differ in genes that modify the expression of the mutant phenotype. This leads to complications when one compares mutants in different backgrounds and in addition gives problems when one combines the new mutant with existing mutants in a different genetic background. This aspect makes it useful to apply mutagenesis on commonly used standard wild types, when there are not specific reasons to use a different parental genotype. In *Arabidopsis* these are Columbia (Col), L*er* and Ws.

3.2 Choice of mutagens

The available mutagens can be divided into physical, chemical, and biological mutagens. The most commonly used physical mutagens are ionizing radiation, such as γ and X-rays, and fast neutrons. Each type of radiation produces deletions at a high frequency. The deletions are supposed to be larger after fast neutron

radiation. The latter type of radiation yields densely ionized tracks compared to the mainly sparse ionizations caused by γ and X-rays. For this reason fast neutrons are sometimes preferred. However, facilities where one can apply this type of radiation are rare in comparison to sources of γ and X-rays. Deletions are attractive for many experiments because the mutants are often true nulls. In addition, a deletion is often recognizable with Southern blot and PCR analysis, which facilitates the recognition of the gene to be cloned using map based cloning (see chapter 6) and genomic subtraction procedures (see chapter 5). Although large deletions, e.g. parts of chromosome arms, can occur they are rarely observed in M2 populations because a large deletion in most cases affects the viability of germinating pollen grains that then will not contribute to fertilization. This effect of reduced transmission of mutated pollen is called certation.

Most chemical mutagens lead to base pair substitutions, especially GC→ AT in the case of ethyl-methane sulfonate (EMS). Physical and chemical mutagens have a different mutant spectrum, which is most obvious when one compares the ratio M1 sterility versus embryo lethal and chlorophyll mutants. For example, in an experiment (10) where EMS and X-rays were compared, an EMS treatment resulting in 44% sterility (percentage reduction of fertilized ovules compared to control) gave 18.2% embryonic lethals and 9.4% chlorophyll mutants, whereas an X-ray treatment that gave almost the same sterility produced only 5.9% embryonic lethals and 1.0% chlorophyll mutants. This relative high sterility is due to the fact that irradiation leads to chromosome breaks and chromosomal aberrations, giving rise to meiotic disturbances and, therefore, sterility. Base pair changes may lead to specific amino acid changes, which may alter the function of proteins, but do not abolish their function as deletions and frame-shift mutations mostly do. Plant species differ in their sensitivity for specific chemicals. In barley, sodium azide is a very potent mutagen (11), but it is hardly effective in *Arabidopsis* (12), where EMS is preferred (see *Protocol 1*). However, several other chemical mutagens have been shown to be effective (1, 2).

Chemical mutagens are extremely toxic and, therefore, require more care in their application, compared with physical mutagens. The latter can also be easily applied to pollen (13, 14). Pollen treatment is attractive for the generation of additional alleles of existing recessive mutants. To achieve this objective wild-type pollen is irradiated and then used to pollinate a mutant. The F1 progeny will be wild type, except when a pollen grain was mutated in that specific gene. In practice, this procedure yields many large deletions, which are not transmitted to the next generations (13, 14, and references therein). The effectiveness of radiation treatments depends heavily on the moisture and oxygen content of the treated material.

Tissue culture can induce so-called somaclonal variation which can be considered in the context of biological mutagens. This method might be useful, when a high enough mutation frequency and preferably, a different spectrum of mutants can be obtained. Although there is no doubt that genuine mutations occur after tissue culture, their frequency is much lower than with classical mutagens. Without question the amount of work to generate large numbers of

M1 plants, or in the case of tissue culture induced mutations R0 (regenerant), R1, or SC1 (somaclonal) plants that subsequently need to be selfed, is much more than a seed treatment with EMS or irradiation. Another negative aspect of this approach is that tissue culture produces many more polyploids in comparison with classical mutagens. A comparison of EMS and tissue culture mutagenesis in tomato has been described by van den Bulk *et al.* (15).

3.3 Mutagen dose

The higher the mutagen dose, the more efficient the experiment, because it provides a higher number of possible mutants. However, there are good reasons not to use the highest mutagen dose possible because this also will lead to an increase in unwanted mutations at other loci, many of which lead to sterility or even lethality. This makes a relatively low dose attractive in those cases where the amount of work and costs involved in growing more M1 plants and screening more M2 plants is not excessive. In those cases where it is expensive to grow many M1 plants and when laborious mutant screens are applied, the costs involved justify a higher dose. This approach then leads to more 'dirty' mutants that need 'cleaning up' by subsequent backcrosses to the wild type.

The determination of the optimal mutagen dose is not easy because of the reasons mentioned above. However, two criteria can be used to access the effectiveness of a treatment. These are the sterility of the M1 plants, which should be significant. Approximate numbers are 30–60% seed set of the control, based on percentage of fertilized ovules, or 20–50% of plants producing no offspring. As mentioned before, one requires considerably higher levels of sterility after irradiation compared to chemical mutagenesis to obtain a reasonable number of mutants. In M1 plants one also should see sectors with pigment defects in up to 1% of the plants. Pigment phenotypes screened for in the M2 population at the seedling level (or within the immature siliques of *Arabidopsis*) have mutation frequencies of 2–15% in populations that have been mutagenized effectively (3, 10). Survival or final germination percentage of the mutagen-treated seeds is not a good criterion because, even in highly mutagenized material, germination is only delayed in *Arabidopsis*. However, when treated seeds are not kept wet enough, germination can be strongly reduced.

Protocol 1

Ethyl-methane sulfonate (EMS) treatment for Arabidopsis

Equipment and Reagents

- Protective clothing and chemical hood
- Buchner vacuum flask
- Cloth bag
- Seeds pre-imbibed in water
- Freshly prepared ethyl-methane sulfonate (EMS) solution of 0.4–0.6% [v/v]
- 1M NaOH
- 0.15–0.2% [w/v] water agar solution

Protocol 1 continued

EMS is highly carcinogenic and volatile. Experiments should, therefore, be performed with all necessary safety precautions such as wearing protective clothing and working in a chemical hood. One should take all required precautions for waste disposal. Decontaminate EMS solutions with 1M NaOH.

Method

1. To allow good uptake of EMS, use seeds pre-imbibed in water. Imbibition at low temperature allows dormancy breaking (when necessary).
2. Prepare from a stock solution a freshly prepared EMS (EMS hydrolyses in water) solution of 0.4–0.6% [v/v]. EMS batches sometimes differ in effectiveness.
3. Stir the solution thoroughly because EMS does not dissolve very well in water.
4. Add a volume of the EMS solution similar to the volume in which the seeds are imbibed so that a 0.2–0.3% [v/v] solution is obtained.
5. Mix the seeds thoroughly with the solution.
6. An alternative procedure (3) is to add the stock solution directly to the seeds imbibed in water to produce a 0.2–0.3% [v/v] solution.
7. Imbibe the seeds for 12–24 hours at room temperature in darkness. The effectiveness of the treatment is the product of EMS concentration $\times$ duration of the treatment (7).
8. Remove the EMS solution by carefully decanting or by passing the seeds over a Buchner vacuum flask. Add a volume of water similar to the volume used for the treatment. Repeat these washes 5–10 times. Collect all EMS and wash waste in a waste container. Add NaOH to the waste to a concentration of 1 M to destroy the EMS.
9. Some researchers (1) pack the seeds in a cloth bag, which facilitates rinsing by transferring the packet of seeds to the wash solutions.
10. Mix the remaining seeds in 0.15–0.2% [w/v] water agar solution, in which the seeds can be pipetted directly to soil. A concentration of 100 seeds per 5 ml is sufficient for 100 cm^2 of soil surface. Keep the seeds moist after planting.
11. An effective EMS treatment will lead to reduced germination speed and reduced seedling growth, the presence of sectors with dominant colour mutations, and growth aberrations and sterility.
12. Procedures for different seed types mainly require adjustment of the treatment and wash volumes for species with large seeds. Planting of individual seeds will mostly be practiced instead of distributing the seeds in liquid agar.

Protocol 2

Radiation mutagenesis of Arabidopsis seeds

Equipment and Reagents

- Non-dormant seeds
- 10 ml 0.15–0.2% [w/v] water–agar in tubes
- Gamma or hard X-rays at a dose between 200 and 300 Gy (10 Gy = 1 Krad)

Method

1. Use non-dormant seeds.
2. Add 1000–5000 seeds to 10 ml 0.15–0.2% [w/v] water–agar in tubes.
3. If dry seeds are the starting material, wait 3–6 hours to allow imbibition of the seeds and then irradiate the tubes with gamma or hard X-rays with a dose between 200 and 300 Gy (10 Gy = 1 Krad).
4. After irradiation, it is better not to store the seeds in the agar solution, dilute the suspension to a density of 100 seeds per 5 ml, which is sufficient for 100 cm^2 of soil surface, and pipette the suspension on moist soil in pots or trays.
5. The seeds will develop into M1 plants, and delayed germination and growth together with sterility will indicate that the treatment was effective.
6. Pollen irradiation can be applied in *Arabidopsis* to inflorescences placed in plastic bags using a dose of 300 and 600 Gy and used immediately after the treatment for pollination (12), preferably of a male sterile genotype. When the pollen carries a dominant marker it allows seeds obtained by crossing from unwanted selfing events to be distinguished.

3.4 Numbers of M1 and M2 plants

Mutation frequencies that are obtained after mutagenesis are often around 5×10^{-4} per locus per genome (7). In cases where the germline contains two diploid cells, with two genomes per cell, there are four genomes per M1 plant. The mutant frequency per M1 plant is then 2×10^{-3}. To obtain a 95% probability of finding a specific mutation in a sample, one requires 1500 M1 plants when the mutant frequency is 2×10^{-3}. Since recessive mutants segregate at a frequency of $^1/_4$ per sector and, therefore, $^1/_8$ per M1 progeny, some mutants will not be found by chance when the M2 population size relative to the number of M1 plants is too small. One can increase the number of mutants by increasing the number of M1 plants and also, to a certain degree, the number of M2 plants. However, it was shown by Rédei (1) that an optimal ratio between the number of M1 plants and the number of M2 plants to be screened exists in order to obtain mutants at the lowest possible costs. This ratio depends on the relative costs of growing the M1 and the M2 plants. When the difference between these is low, screening a small number of M2 plants per M1 progeny is most cost effective.

This is often the case in *Arabidopsis*, where researchers prefer growing large numbers of M1 plants and screen between 2000 and 125 000 (3) M2 plants, depending on the ease of the screen. When one screens 10 000 M2 plants, with the assumption that no M1 plants are represented more than once, which is the case with very high numbers of M1 plants used to obtain this M2, this gives $\frac{1}{8} \times 2 \times 10^{-3} \times 10\,000 = 2.5$ mutants per locus. In cases where growing M1 plants and harvesting M2 seeds is relatively expensive, for example, as in tomato, it is worth increasing the number of M2 plants per M1 plant in order not to miss any mutants by chance. This is especially relevant when M2 screening can be done at the seedling stage.

In maize, 1000 non-chimeric M1 plants obtained after pollen mutagenesis was suggested to be sufficient to obtain 1 recessive mutant per locus (6).

3.5 The handling of M1 plants

For *Arabidopsis*, M1 plants are often grown in large numbers per pot or flat, and harvesting these as bulk or in pools is most efficient. However, pooling M1 plants implies that when a mutant is sterile or lethal it is lost. Furthermore, finding a mutant at the same locus twice may be because the two mutants derive from the same cell and, therefore, the same mutation event. Harvesting individual M1 plants and keeping spare seeds will allow the recovery of heterozygous wild type sister-plants, which are within the M1 progeny with twice the frequency of the recessive mutant. Furthermore, finding the same mutant in different progenies provides unambiguous proof for them originating from independent mutation events. Pooling the progenies of a certain number of plants provides a compromise between harvesting individual M1 plants, also called 'pedigreeing' (3) and harvesting all M1 plants as a bulk. However, for efficient recovery of heterozygous sister-plants the number of M1 plants per pool will quickly become too large and, therefore, 'pedigreeing' is necessary for this purpose. To be sure that a sufficient number of independent mutants are isolated, pools of up to a few hundred M1 plants are efficient. These numbers are often obtained when all M1 plants in one pot or tray are harvested. When two phenotypically identical mutants are found in one pool, they are often considered to be derived from the same mutation event. Only one mutant will then be used for further analysis. For large seeded plants one mostly collects single M1 progenies, for example as single maize cobs.

4 Further analysis of mutants

Since mutant screens depend on what the individual researcher is looking for the M2 screening methods can differ very much. These will not be discussed as part of this chapter.

However, when a certain mutant is isolated, a number of generally applicable procedures are required for their standard genetic analyses, which are described in more detail elsewhere (16, 17).

The main points are summarized below:

- In case the mutant is sterile, which mostly is due to male sterility, pollination with wild type pollen will save the mutant. Male sterility is indicated by the absence or poor quality of pollen. When the pollen of a sterile plant looks fertile, it can be used to pollinate a wild type plant. Female sterility is often observed in megaspore and ovule mutants. Harvesting fertile (heterozygous) sister-plants from the same M2 family also helps to save the mutant.
- Confirm the mutant phenotype in the M3 generation or in the generation derived from selfing the hybrid obtained by crossing the mutant with wild type or from the heterozygous sister-plant.
- Determine the segregation ratio of the mutant by crossing it with the parental wild type and by analysing the F2 generation. A backcross of the F1 to the recessive parent to analyse the genetics of the mutant is also valuable. The analysis of a segregating population, in addition, allows the analysis of putative pleiotropic effects observed in the selected mutant. Absolute linkage of different characters associated with the mutant will indicate true pleiotropism, as well as observing the same association of characters in independent allelic mutants. Segregation of the various traits implies that mutations in different genes are responsible for the syndrome observed in the original mutant. The phenotype of the F1 will already show whether the mutant is dominant, recessive, or co-dominant.
- Cross the mutant with all available mutants that have a similar phenotype. The absence of complementation with an existing mutant indicates allelism in the case of recessive mutants. For dominant mutants, the segregation of wild type plants in the F2 progeny will indicate non-allelism.
- When a new locus is found, its map position should be determined. A specific location on the genetic map may also suggest that other previously isolated mutants, even those with a (slightly) different phenotype, may represent mutants at the same locus, which can be confirmed with allelism tests.

References

1. Rédei, G. P. and Koncz, C. (1992). In *Methods in Arabidopsis research* (ed. C. Koncz, N-H. Chua, and J. Schell), p. 16. World Scientific Publishing, Singapore.
2. Feldmann, K. A., Malmberg, R. L., and Dean, C. (1994). In *Arabidopsis* (ed. E. M. Meyerowitz and C. Somerville), p. 137. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
3. Lightner, J. and Caspar, T. (1998). In *Methods in molecular biology: Arabidopsis protocols* (ed. J. Martinez-Zapater and J. Salinas), Vol. 82, p. 91. Humana Press Inc, Totowa, NJ.
4. Van Harten, A. M. (1998). *Mutation breeding: theory and practical applications*. Cambridge University Press, Cambridge.
5. Li, S. L. and Rédei, G. P. (1969). *Radiat. Bot.*, **9**, 129.
6. Neuffer, M. G., Coe, E. W., and Wessler S. R. (1997). *Mutants of maize*, pp. 396–401. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
7. Koornneef, M., Dellaert, L. M. W., and van der Veen, J. H. (1982). *Mutation Research,* **93**, 109.

8. Yonezawa, K. and Yamagata H. (1975). *Radiat. Bot.,* **15**, 241.
9. Alonso-Blanco, C. and Koornneef, M. (2000). *Trends Plant Sci.*, **5**, 22.
10. Van der Veen, J. H. (1966) *Arabidopsis Information Service*, **3**, 26.
11. Kleinhofs, A., Owais, W. M., and Nilan, R. A. (1978). *Mutat. Res.*, **55**, 165.
12. Gichner, T. and Veleminsky, J. (1977). *Biologia. Plantarum (Praha)* **19**, 153.
13. Liharska, T.B., Hontelez, J., van Kammen, A., Zabel, P., and Koornneef, M. (1997). *Theor. Appl. Genet.*, **95**, 969.
14. Vizir, I. Y., Anderson, M. L., Wilson, Z. A., and Mulligan, B. J. (1994). *Genetics*, **137**, 1111.
15. Van den Bulk, R. W., Löffler, H. J. M., Lindhout, W. H., and Koornneef, M. (1990). *Theor. Appl. Genet.*, **80**, 817.
16. Meinke, D. and Koornneef, M. (1997). *Plant J.*, **12**, 247.
17. Koornneef, M., Alonso-Blanco C., and Stam, P. (1998). In *Methods in molecular biology: Arabidopsis protocols* (ed. J. Martinez-Zapater and J. Salinas), Vol. 82, p. 105. Humana Press Inc, Totowa, NJ.