INTRODUCTION

Abiotic stress, such as high salt or low temperature, adversely affects plant growth and development. Salt stress inhibits seed germination, retards plant growth, and accelerates senescence. Freezing or drought stress can cause cell damage and plant death. The following parameters can be used to evaluate plant tolerance to salt, drought, or freezing stress: root elongation, fresh weight gain, seed germination (described here), electrolyte leakage, or water loss measurement. Several stress mutants have been characterized using these tests, including hos1 and hos2, which show higher expression of some stress-regulated genes when exposed to low-temperature stress; hos5, which shows higher expression of some stress-regulated genes under abscisic acid (ABA) and salt treatments; sfr mutants, which are deficient in freezing tolerance; and eskimo1, which is constitutively freezing-tolerant. This protocol describes a germination assay that can be carried out on seeds subjected to osmotic or hormone-induced stress. The seeds are plated on filter paper saturated with ABA (for hormonal stress) or with NaCl or mannitol (for osmotic stress). The germination rate can be scored on different days; germination is considered to have occurred when the radicles have penetrated the seed coats. The levels of stress suggested in this protocol may need to be adjusted, depending on the ecotype and growth conditions used.

RELATED INFORMATION

For information about obtaining a mutant in Arabidopsis, see Setting Up Arabidopsis Crosses (Weigel and Glazebrook 2006), Genetic Analysis of Arabidopsis Mutants (Weigel and Glazebrook 2008), and the Discussion. For further information about stress mutants with altered tolerances, see Warren et al. (1996), Ishitani et al. (1998), Xin and Browse (1998), Lee et al. (1999), and Xiong et al. (1999).

MATERIALS

Reagents

Filter paper, saturated with one of the following:

- ABA (0-5 μM in H₂O)
- H₂O
- Mannitol (0-500 mM in H₂O)
- NaCl (0-50 mM in H₂O)

Cut filters to fit over the surface of the MS plates.

- MS medium (1X), in plates

Seeds of interest

As controls, use aba and abi mutants, which are defective in ABA biosynthesis and ABA signaling, respectively. They
are available from the Arabidopsis Biological Resource Center (ABRC).

**Equipment**

Aluminum foil

Incubator (long-day or continuous-light)

**METHOD**

1. Surface-sterilize at least 100 seeds of each mutant line plus wild type. Include additional control mutants as appropriate.

2. Sow and incubate the seeds as follows:
   i. Lay paper filters saturated with the appropriate solution on the surface of MS plates.
   ii. Sow the seeds on filter paper at a density of 100 per plate.
   iii. Wrap the plates in aluminum foil.
   iv. Incubate for 48 h at 4ºC.

3. Remove the aluminum foil and transfer the plates to a long-day or continuous-light incubator.

4. Score the germination rate, as a percentage of seeds sown, for each group of plants after 10 d.

**DISCUSSION**

Two methods of obtaining a mutant are by directed screening for plants with a certain phenotype and by knocking out a gene of interest. The phenotypes of knockout mutants are difficult to predict, and it is also quite common for mutants, first isolated because of a specific phenotype, to have other, pleiotropic defects, i.e., multiple defects in addition to that of primary interest. Thus, it is often necessary to characterize a variety of phenotypic parameters. This is particularly important as the field of molecular genetics expands. If a number of groups, each looking for a different phenotype, independently identify different mutations in the same gene, an awareness of additional defects will often show that the new mutation is allelic to existing mutants.

Before embarking on an extensive phenotypic characterization, it is important to confirm that the various phenotypes of an individual are genetically linked (see Setting Up Arabidopsis Crosses [Weigel and Glazebrook 2006] and Genetic Analysis of Arabidopsis Mutants [Weigel and Glazebrook 2008]). Strictly speaking, all of the phenotypes should be mapped, but it is normally sufficient to backcross a newly isolated mutant about five times to remove defects due to unlinked mutations. Phenotype mapping becomes more important, however, when dealing with a mutant that has been isolated by site-selected mutagenesis (T-DNA or transposon knockout). In this case, it must be shown that any phenotype cosegregates with the induced mutation. Definitive proof that a phenotype is indeed due to the disrupted gene can only come from complementing the mutant with a wild-type copy of the disrupted gene or by constructing trans-heterozygotes carrying two independently isolated mutations.

**REFERENCES**


**Recipe**

**MS medium (1X)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount to add to make 1 L</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS salts</td>
<td>4.33 g</td>
</tr>
<tr>
<td>Bacto agar</td>
<td>7 g</td>
</tr>
<tr>
<td>H₂O</td>
<td>To make 1 L</td>
</tr>
</tbody>
</table>

Combine MS salts and H₂O, and stir to dissolve. Adjust pH to 5.7 with KOH. Add agar. Sterilize by autoclaving. Plants grow more quickly in MS containing 1% (w/v) sucrose, but so do microorganisms: Including sucrose in the medium significantly increases the chance of microbial contamination.

**Topic Introduction**

**Genetic Analysis of Arabidopsis Mutants**

Detlef Weigel and Jane Glazebrook


**INTRODUCTION**

The inheritance of mutant phenotypes in Arabidopsis thaliana is most often analyzed in the progeny resulting from crosses between different parents. This article describes genetic strategies for the analysis of mutants. Newly identified mutations can be examined initially using segregation analysis, followed by backcrossing and cosegregation for removal of extraneous mutations and assessment of pleiotropy. Confirmation that a phenotype of interest results from a given mutation can be achieved via complementation testing, which is also used to determine allelism of recessive mutations. The construction of double mutants is useful in the study of signal transduction and metabolic pathways. Considerations for the use of double homozygotes and their identification in the F₂ and F₃ generations are described at the end of this article.

**SEgregation ANALYSIS**

Segregation analysis is used to determine whether a mutant phenotype is caused by dominant or recessive mutations, how many mutations are required for the phenotype, and whether the mutations are in cytoplasmic or nuclear genes. With a newly identified mutation, it is advisable to examine the segregation pattern of the phenotype first in the self-progeny, then in the F₁ of a cross to wild type and in the F₂ progeny, and finally in several backcrosses to wild type to
remove extraneous mutations.

**Self-Progeny**

Recessive mutations are first identified in mutant screens as homozygotes, and, if the mutant phenotype is fully penetrant, each of the self-progeny of such plants will be mutant. Dominant mutations can be identified as heterozygotes or homozygotes. If heterozygous, one quarter of the self progeny will exhibit a wild-type phenotype (Aa x Aa → 1/4 AA: 1/2 Aa: 1/4 aa). In this situation, homozygous mutants can be identified as plants that are true breeding for the mutation (i.e., do not yield wild-type offspring). It is most convenient to use homozygous mutant plants for subsequent segregation analyses.

**F1 Progeny**

To determine whether the mutation can be transmitted through both male and female gametes, it is important to carry out reciprocal crosses, pollinating wild-type carpels with mutant pollen and mutant carpels with wild-type pollen. If the resulting F1 plants are wild type, then the mutation is nuclear and recessive; if mutant, then the mutation is nuclear and dominant. If the F1 progeny from the cross between wild-type pollen and mutant carpels show the mutant phenotype, but progeny from the reciprocal cross do not, the mutation may be encoded by the mitochondrial or plastid genomes or by some other maternal factor (cytoplasmic inheritance). In any event, collect seed from individual F1 plants (F2 seed) for further analysis.

**F2 Progeny**

Grow approximately 100 F2 plants and determine what fraction of the plants exhibit the mutant phenotype. If the mutant phenotype is caused by a single, recessive, nuclear mutation, one-quarter of the plants will display the mutant phenotype. If it is caused by a single, dominant, nuclear mutation, three-quarters of the plants will show the mutant phenotype. In some cases, heterozygous plants exhibit a phenotype intermediate between those of the two homozygous genotypes. This is called semidominance. In this case, three classes of plants will be observed in the progeny, in the ratio 1:2:1. If other, more complicated, genotypes are responsible for the mutant phenotype, different segregation patterns will be observed, e.g., two unlinked recessive mutations (1/16 mutant phenotype); one recessive and one unlinked dominant mutation (3/16 mutant phenotype); two unlinked dominant mutations (9/16 mutant); either one of two unlinked recessive mutations (7/16 mutant), etc.

The Chi-square ($\chi^2$) statistical test can be used to determine how well a set of segregation data fits a particular hypothesis. The formula is:

$$\chi^2 = \sum \frac{(O - E)^2}{E}$$

where $O$ is the observed number of plants in a category and $E$ is the expected number of plants in a category. $O$ and $E$ are the actual number of plants scored, rather than a percentage or ratio. This means that the significance of the results increases with the number of plants analyzed. The classes are the different phenotypic groups. The degrees of freedom ($df$) = (number of classes) – 1.

For example, an F2 population consists of 21 phenotypically mutant plants and 79 phenotypically wild-type plants. Is this result reasonable if the phenotype is caused by a single recessive mutation? As there are two phenotypic classes, there is 2 – 1 = 1 degree of freedom. For a single, recessive gene, one expects 25 mutant and 75 wild-type plants, so, using the observed data,

$$\chi^2 = \frac{(21 - 25)^2}{25} + \frac{(75 - 79)^2}{75} = 0.64 + 0.21 = 0.85$$

Generally, a hypothesis is considered acceptable if the calculated value of $\chi^2$ is less than the value for $\chi^2_{0.95}$. In this example, with one degree of freedom, $\chi^2_{0.95} = 3.841$. This means that the data fall well within the range that is to be expected for 95% of sample sets occurring from segregation of single recessive genes. So we can accept our hypothesis that the results are reasonable for 3:1 segregation.
In contrast, if we had postulated that the phenotype was caused by two unlinked recessive genes, 1/16 mutant plants would have been expected, and $\chi^2$ would have been:

$$\chi^2 = \frac{(21 - 6.25)^2}{6.25} + \frac{(79 - 93.75)^2}{93.75} = 34.81 + 2.32 = 37.13$$

This is well above 3.841, so this hypothesis would have been rejected. For a more detailed description of $\chi^2$ tests, consult a statistics text (e.g., Remington and Schork 1985).

Note that there is often more than one hypothesis that fits segregation data. Our example also passes a $\chi^2$ test of the hypothesis that the phenotype requires two genes, one dominant and one recessive (3/16 mutant phenotypes expected). To distinguish between hypotheses that predict very similar segregation ratios, large numbers of F2 plants must be scored. If the data fit a simple segregation hypothesis, then that hypothesis is generally accepted, unless there is some reason to believe that the genetics are more complicated. Moreover, when complex inheritance patterns are suspected, stronger evidence can be obtained by determining if F3 individuals show the expected phenotypes.

Often, the first backcross of a newly identified mutant yields fewer mutant plants in the F2 generation than expected. This is not necessarily an indication of complicated genetics. Skewed ratios can be caused by a number of phenomena, including the segregation of multiple, unrelated mutations in a mutagenized background. For example, the original isolate may carry a second, linked mutation, with deleterious effects on survival or growth, causing the mutant phenotype to be underrepresented in the F2 population. Before concluding that a phenotype is caused by something more complicated than a mutation in a single gene, it is wise to backcross the mutation a few times to see if the peculiar segregation ratio persists.

Skewed segregation ratios can also be caused by factors such as lethality in the male or female gamete, poor transmission through the male or female gamete, incomplete penetrance of the phenotype, epigenetic effects, and maternal effects.

**BACKCROSSES AND COSEGREGATION**

As noted above, plants from mutagenized populations frequently carry multiple mutations. Some of these may affect the phenotype of interest. It is advisable to backcross mutant plants to their wild-type parents several times to remove extraneous mutations. Each time a mutant is crossed to wild type, one-half of the mutant genome is replaced with wild-type genes. Subsequent analysis of the F2 population allows the plants that are homozygous for the recessive mutation of interest to be recovered, but does not increase the likelihood of removing unlinked secondary mutations because only one-quarter of the plants will have lost such a mutation (an "improvement"), whereas one-half of the plants will be heterozygous and one-quarter of the plants will be homozygous for the secondary mutation. Consequently, each backcross removes half of the unlinked secondary mutations, and the probability that a particular unlinked mutation remains after $n$ rounds of backcrossing is $(1/2)^n$. The chance of such a mutation remaining after four rounds of backcrossing is 0.062 (i.e., 0.54). Of course, if a secondary mutation is linked to the mutation of interest, the probability of removing it in a backcross is much less than one-half and approaches zero as the distance between the primary and secondary mutations decreases.

Confirmation that a phenotype of interest is due to a particular mutation can be obtained by identifying the corresponding wild-type gene, introducing that gene into mutant plants, and demonstrating that the mutant phenotype is rescued (complementation). In the case of dominant mutations, it is necessary to demonstrate that a transgene construct carrying the dominant allele transforms wild-type plants to the mutant phenotype.

If a mutant exhibits two or more phenotypes, it is usually necessary to determine whether both phenotypes result from the same mutation (pleiotropy) or from two or more different mutations. These possibilities can usually be distinguished by multiple rounds of backcrossing or by testing for cosegregation of the phenotypes, which is generally much faster.

Consider the probability ($p$) of phenotypes caused by two unlinked recessive mutations cosegregating. The probability that they are not caused by two unlinked, recessive mutations is $1 - p$. Among the F2 progeny of a backcross to wild type, if the two mutations are recessive and unlinked, the probability of a plant that is homozygous for one mutation (genotype $aa$ showing phenotype "a") also being homozygous for the second mutation (genotype $bb$, hypothetical
cause of phenotype "b") is one-quarter. The probability that all of \( n \) plants that display phenotype "a" will also display phenotype "b" is \( 1/4^n \). For example, the probability that two recessive phenotypes, which cosegregate among four plants, are caused by two unlinked mutations is 0.004 (i.e., \( 0.25^4 \)); the probability that this is not true is 0.996 (i.e., \( 1 - 0.004 \)). Thus, the phenotypes are almost certainly caused by the same mutation, or by linked mutations.

Of course, the possibility that the two phenotypes are caused by two linked mutations, rather than one single mutation, is much more difficult to exclude, and the difficulty increases with decreasing distance between the mutations. (The probability that \( aa \) and \( bb \) will cosegregate is \( (1 - r)^{2n} \), where \( r \) is the recombination frequency between the two genes.) Consider two genes with 1% recombination between them that are presumed to account for two phenotypes. To test this hypothesis, enough plants must be examined to be 95% confident that a recombinant has been recovered. If the recombinant has both phenotypes, then these phenotypes must be caused by a mutation in only one gene (\( aa \)); on the other hand, if one recombinant is found that lacks one of the phenotypes, then the phenotypes are caused by mutations in two genes (\( aa \) and \( bb \)).

How many plants are enough? The probability of finding a recombinant (i.e., that \( aa \) and \( bb \) will not cosegregate) is \( 1 - (1 - r)^{2n} \), and a 95% probability of finding a recombinant is reached when \( 0.95 = 1 - (0.99)^{2n} \). Solving for \( n \) yields:

\[
0.05 = -(0.99)^{2n}
\]

\[
\log[0.05] = 2n \log(0.99)
\]

\[
\frac{\log[0.05]}{\log(0.99)} = 2n
\]

or \( n = 149 \) plants.

In the case of insertion mutations, it is desirable to determine whether the insertion caused the mutant phenotype. The situation is complicated by the fact that insertion mutants often have insertions at more than one site. If the insertion is tracked by a dominant drug resistance phenotype, and the mutant phenotype is recessive, many plants that display the drug resistance will not display the mutant phenotype, even if one of the insertions causes the phenotype. However, all plants that display the mutant phenotype must carry this insertion. If the mutant phenotype is recessive, and there is one unlinked insertion, then the probability that \( n \) plants displaying the mutant phenotype will also display drug resistance is \((3/4)^n\), or 0.056 for 10 plants. If there are two unlinked insertions, the probability is \((15/16)^n\) or 0.040 for 50 plants.

Obviously, the possibility that a linked insertion does not cause the phenotype is much more difficult to exclude. Now that isolation of sequences adjacent to insertion sites has become straightforward, many investigators prefer to test whether a particular insertion caused the phenotype by introducing the corresponding wild-type sequence into the mutant plants and testing for complementation of the mutant defect.

**COMPLEMENTATION TESTING**

If two mutations cause similar phenotypes, and they are both recessive, complementation testing can be used to determine whether they are alleles of the same gene. The two homozygous mutants are crossed to each other, and the phenotype of the \( F_1 \) progeny is examined. If the mutations are allelic, the cross would have been \( a_1/a_2 \times a_2/a_2 \rightarrow a_1/a_2 \), and the progeny would show the phenotype of the mutant parents (they fail to complement). If the mutations are alleles of different genes, then the cross would have been \( aaBB \times AAbb \rightarrow AaBb \), and the \( F_1 \) plants would show the wild-type phenotype (they complement). It is advisable to include a second mutation in complementation testing crosses that can be used to distinguish cross from self-progeny, since otherwise there is no way to distinguish noncomplementation from selfing of the female parent. There are instances of two mutations appearing to be recessive, but failing to complement even though they are in different genes. This can occur if the two genes act in the same pathway, and a recessive allele of one does not cause a noticeable effect, but the combination of two recessive alleles in two different genes does cause a noticeable effect. This phenomenon can be detected by observing recombination frequencies in the \( F_2 \). If the mutations are alleles of the same gene, then all of the \( F_2 \) progeny of an \( a_1/a_2 \) plant will show the mutant phenotype. Conversely, if the mutations are in two unlinked genes, the \( F_2 \) progeny will segregate 5:11 wild-type:mutant.
Complementation testing cannot be used to determine allelism of dominant mutations. It is possible to use recombination testing for this purpose. The homozygous mutant plants are first crossed, the F₁ is allowed to set seed, and the phenotypes of the resulting F₂ are examined. If two dominant mutations are unlinked, 1/16 of the progeny will be homozygous for the recessive (wild-type) alleles of both mutations. If wild-type plants are not observed, either the mutations are allelic or the genes are linked.

If there is a possibility that a recessive mutation is an allele of a gene whose DNA sequence is known, complementation testing can be done by generating transgenic plants. The wild-type version of the suspect gene is cloned into a transformation vector and introduced into homozygous mutant plants. If the mutation is an allele of the transgene, the transformed plants will exhibit the wild-type phenotype. This approach is especially useful in cases when other alleles of the suspect gene are not available. As a shortcut, one can first determine whether the suspect gene carries a sequence change in the mutant by PCR amplifying the gene from mutant DNA and sequencing it.

STRATEGIES FOR IDENTIFYING DOUBLE MUTANTS

Double mutants are valuable tools for studying signal transduction and metabolic pathways. The phenotypes of double mutants can be used to determine whether genes act in different pathways or in the same pathway, the order in which genes act in a pathway, and whether they have redundant functions. The most efficient strategy for constructing a double mutant depends on how much is known about the two mutations of interest. This discussion will assume that a double homozygote is desired, even if one of the mutations is dominant, as a double homozygote can be easily propagated by selfing. Before beginning, it is wise to consider the following questions:

- Are molecular (preferably PCR) markers available that are tightly linked to the mutations? Such markers are very helpful because they allow the genotype to be determined without reference to phenotype; the phenotypes of double mutants can be difficult to predict.

- Are the mutations recessive or dominant? This affects the expected frequencies at which plants with the mutant phenotypes appear in F₂ populations, and the ease with which homozygous plants can be identified.

- Are the genetic map positions of the mutations known? Are they linked? If the two mutations are linked, the frequency of the double homozygotes in the F₂ drops precipitously with decreasing genetic distance.

- Is the phenotype of the double mutant predictable? Is it readily distinguishable from each homozygous single mutant? If the phenotype is not predictable, or is expected to be very similar to one of the single mutants, double mutants cannot be identified by phenotype alone.

Finding Double Mutants in the F₂ Generation

Identifying double mutants in the F₂ generation is a simple method (see below) that works well if both mutations of interest are recessive and unlinked, and if either the phenotype of the double mutant is predictable and different from that of each single homozygote, or if the mutations can be identified using molecular markers.

Method

1. Cross the homozygous mutants to each other.

2. Allow the F₁ to self, and plant the F₂ seed.

3. If the double mutant has a predictable phenotype, look for plants with that phenotype (e.g., if crossing gl1 and ap2, look for plants without trichomes [gl1] and petals [ap2]). If both mutations are detectable with molecular markers, use these to find the double homozygote.

Requirements

Examine enough plants to ensure that the double mutant is identified. The probability of not finding the mutant is $(15/16)^n$ (where $n$ is the number of plants to be tested). Thus, the probability of not finding the double mutant has decreased to 5% when $n = 47$ since $(15/16)^{47} = 0.05$. A convenient rule of thumb for these types of calculations is that a 95% confidence level is reached when the sample size is approximately three times larger than the average
number of expected events. In this case, the expected frequency of finding one double homozygote in the F2 is 1 in 16 total plants. Thus, to be 95% confident of finding a double homozygote, \((\sim3)(16) = \sim48\) plants must be tested.

**Finding Double Mutants in the F3 Generation**

There are several situations in which it can be very helpful to look for double mutants in the F3 generation. One such situation occurs when the mutations of interest are linked. If the recombination frequency between two mutations is 10%, the frequency of the double mutant in the F2 is (the probability of finding an F2 homozygous for the first gene) (the probability of cosegregating the linked mutation on both chromosomes) = \((1/4)(0.1)(0.1) = 0.0025\), or 1 plant in 400. As described above, to be 95% confident of finding the double homozygote \((aabb)\), \((2.94)(400) = \sim1200\) plants must be examined.

In this same F2 population, however, the frequency of the \(aa\) genotype is 0.25, and 19% of \(aa\) plants are \(bb\) or \(bb\) \([(0.1)(0.1) = 1\% are bb; (2)(0.1)(0.9) = 18\% are \(bb\); (0.9)(0.9) = 81\% are \(BB\)]. One option then is to identify \(aa\) homozygotes in the F2 and look for \(bb\) homozygotes in the F3. Because one-fifth of all F2 families carry a \(b\) allele, there is a 95% probability that among approximately \((3)(5) = 15\) \(aa\) F3 families, at least one will segregate \(bb\) plants. This strategy is particularly appealing if the phenotype of \(aa\) is difficult to score, and the phenotype of \(bb\) is easy to score.

F3 strategies are also useful when there are no molecular markers for the mutations, and the phenotype of the double mutant cannot be predicted. Consider an example in which there are two recessive mutations of interest, one that causes constitutive expression of a normally inducible reporter gene \((aa)\), and one that blocks inducible expression \((bb)\). The investigator suspects that the mutations each define a regulatory gene and that they act in the same pathway. If A acts before B, the prediction is that the \(aabb\) double mutant will have the phenotype "b" characteristic of the \(bb\) genotype, and if B acts before A, the double mutant will have the phenotype "a", characteristic of genotype \(aa\). The goal is to find the double mutant without assuming its phenotype. In the F2, collect seed from individual plants with the "a" phenotype \((aa\) genotype) and plants with the "b" phenotype \((bb\) genotype). Examine the phenotypes of plants in the F3 families; those F2 plants that were true breeding were homozygous for one or both of the mutations. If the phenotype of \(aabb\) is "b", then none of the F2 plants scored as "a" will have been \(bb\). However, two-thirds of them carried a \(b\) allele \((Bb)\). The F3 seed from these \(aaBb\) plants will segregate in a ratio of 3 "a" phenotypes to 1 "b" phenotype. These phenotypically "b" plants are \(aabb\), and the investigator can conclude that \(bb\) suppresses \(aa\), consistent with the idea that B acts downstream from A. If the phenotype of \(aabb\) is the same as "a", the plants scored as "a" in the F2 will not give rise to "b" plants in the F3, but some of the plants scored as "b" in the F2 will segregate "a" plants in the F3.

**The Brute Force Approach**

Brute force can be used when there are no molecular markers for either mutation of interest, nothing is known about the phenotype of the double mutant, the mutations are recessive, or the mutations are dominant. The only important factor for this approach is that the mutations are not linked. If they are linked, the amount of work involved becomes impractical. As explained above, the frequency of the double mutant in the F2 population resulting from a cross of the two homozygotes is 1/16. To be 95% confident of finding the double homozygotes, 48 plants must be tested. If the mutations are recessive, each of 48 F2 plants must be crossed to both homozygous parents. The plants that fail to complement both parents will be the double homozygotes. For each cross to the parents, several progeny should be tested to ensure that heterozygous F2 plants are not confused with homozygotes. It is very important not to make a mistake here, so it is best to impose a minimum confidence level of 99% (sevenfold higher than the average), reached at seven F3 plants. If one or more of the mutations of interest is dominant, the F2 plants must be crossed to wild-type plants, and the mutant phenotype searched for in the resulting progeny. This approach can be used in combination with other approaches. For example, if there is a molecular marker for mutation \(a\), it can be used to identify \(aa\) plants. These \(aa\) plants can be crossed to \(bb\) plants to reveal which of the \(aa\) F2 plants were also \(bb\).

If uncertain about the genotypes of putative double mutants, it is a good idea to cross the putative double mutant to both single mutant parents (or wild type, if one of the mutations is dominant) to confirm that the putative double mutant really is the double homozygote.
REFERENCES