Plant Breeding Methods

Plant Breeding Methods

Walter R. Fehr and Walter P. Suza

Iowa State University Digital Press
Ames, Iowa

This book is published by

Iowa State University Digital Press
701 Morrill Rd, Ames, Iowa 50011, United States

www.iastatedigitalpress.com

DOI: https://doi.org/10.31274/isudp.2024.159

Iowa State University is located on the ancestral lands and territory of the Baxoje (bah-kho-dzhe), or Ioway Nation. The United States obtained the land from the Meskwaki and Sauk nations in the Treaty of 1842. We wish to recognize our obligations to this land and to the people who took care of it, as well as to the 17,000 Native people who live in Iowa today.

Contents

Introduction

Welcome to *Plant Breeding Methods*, a textbook designed to equip you with the skills needed to successfully manage a cultivar development program. Throughout this textbook, you will study the strategies used by plant breeders to develop clonal, pure-line, hybrid, and synthetic cultivars. By the end, you will have a solid understanding of the different breeding methods and the ability to design your own breeding program for any of the cultivar types.

This textbook covers nine essential topics that are crucial to the development of cultivars. These topics include: (1) Types of cultivars and modes of plant reproduction, (2) Sources of parental germplasm, (3) Population formation by hybridization, (4) Techniques for artificial hybridization, (5) Mutation breeding, (6) Molecular markers and genetic engineering, (7) Backcrossing, (8) Inbreeding, and (9) Recurrent selection. These topics will serve as the foundation for a detailed study of the development of clonal, pure-line, hybrid, and synthetic cultivars in *Principles of Cultivar Development*.

Types of Cultivars and Modes of Plant Reproduction

Walter R. Fehr and Walter P. Suza

Readings:

- Chapter 29: Types of Cultivars [pdf], *Principles of Cultivar Development. Vol. 1: Theory and Techniques*, by Walter R. Fehr (Access the full book)

Introduction

The overall objective of the plant breeding courses is to teach you how breeders develop cultivars of plant species. The methods that breeders use depend directly on the type of cultivar used to produce a commercial crop. Therefore, the purpose of this lesson is to understand the four most common types of cultivars. A **cultivar** is defined as a group of plants that have certain genetic, morphological, and physiological features that distinguish them from other groups of plants within a species. The four most common types of cultivars are (a) clonal cultivars (b) synthetic cultivars (c) pure-line cultivars and (d) hybrid cultivars.

Modes of cultivar reproduction

Plant species can be reproduced sexually, asexually, or by both modes. **Sexual reproduction** occurs when the nucleus of a pollen grain unites with the egg cell in the ovary to produce the embryo of a seed. **Asexual reproduction** represents the propagation of an individual from its somatic tissue.

Cultivar homozygosity vs. heterozygosity

The terms homozygosity and heterozygosity refer to the genetic makeup of an individual plant in a cultivar. Plants are considered **homozygous** when the alleles at a locus are the same. They are considered **heterozygous** when the alleles at a locus are different. The primary method of achieving homozygosity is by self-pollination of individuals, which is a routine part of developing pure-line cultivars or inbred lines used to produce a hybrid. Heterozygosity results from crossing plants with different genotypes. The crossing may be done by hand or through open pollination by wind or insects. Plants in a clonal, synthetic, or hybrid cultivar are heterozygous.

Cultivar homogeneity vs. heterogeneity

The terms homogeneity and heterogeneity refer to the genetic and phenotypic relationship among plants in a cultivars. A cultivar is **homogeneous** when its plants are genetically and phenotypically identical and **heterogeneous** when its plants are genetically different.

Types of Cultivars

Clonal cultivars

Clonal cultivars are reproduced asexually from a single plant that the breeder has selected. As a result, all of the plants in a clonal cultivar are genetically identical or homogeneous. The individual plants in a clonal cultivar are heterozygous because no inbreeding is involved in developing a population for selection. Methods of clonal propagation include cuttings, tubers, bulbs, rhizomes, grafts, and buds. Seed produced through apomixis also is a form of asexual reproduction.

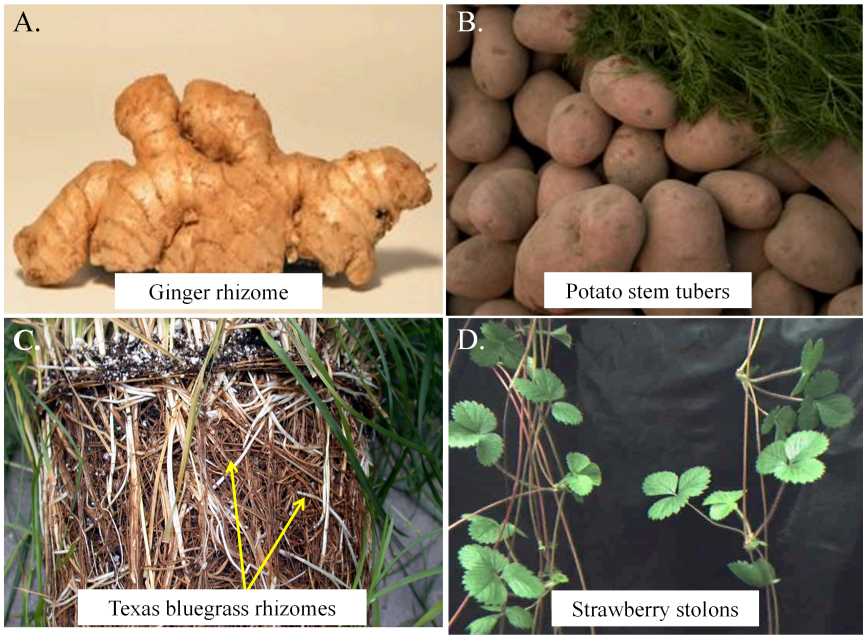


Figure 1. Examples of vegetative tissue used for the propagation of clonal cultivars. (A and B) obtained from Stock.xchng at http://www.sxc.hu/. (C and D) courtesy of Shui-zhang Fei, Iowa State University.

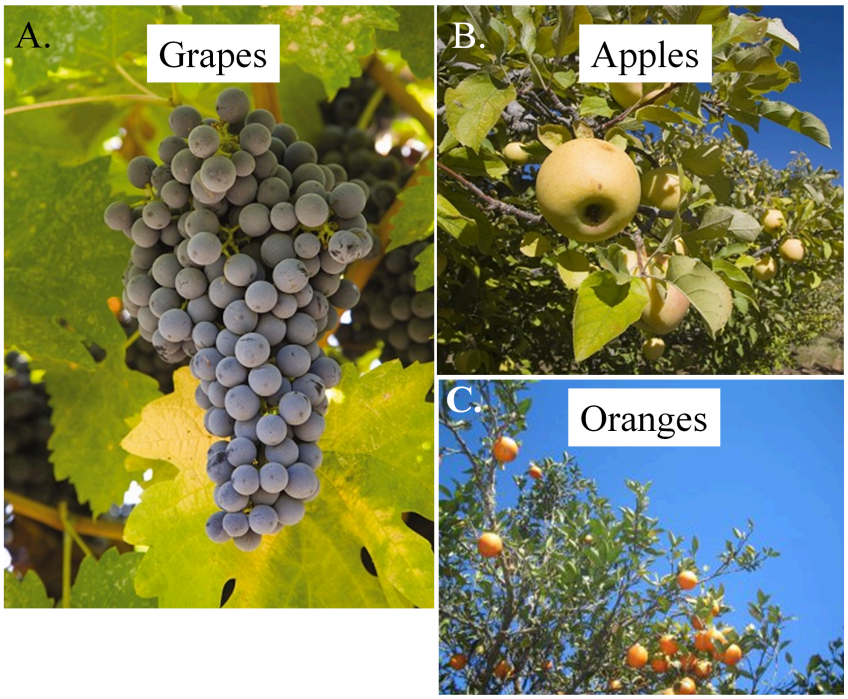


Figure 2. Examples of plant species for which clonal cultivars are used for commercial production. (A and B) courtesy of USDA NRCS. (C) obtained from Stock.xchng at http://www.sxc.hu/.

Synthetic cultivars

The seed of a synthetic cultivar is produced sexually by open pollination. As a result of open pollination, the plants in a commercial field of a synthetic cultivar are heterozygous and heterogeneous.

Figure 3. An example of plant species for which synthetic cultivars are used for commercial production. Photo courtesy of Lynn Betts, USDA NCRS.

Pure-line cultivars

The seed of a pure-line cultivar is produced by self-pollination. As a result, the individual plants are considered to be homozygous and genetically similar to each other or homogeneous. The seed of a pure-line cultivar harvested in one season is used to plant commercial fields the next season.

Figure 4. Examples of pure-line cultivars. Rice photo courtesy of Gary Cramer, USDA NRCS; Wheat photo courtesy of Jeff Vanuga, USDA NRCS, Tobacco photo obtained from Stock.xchng at http://www.sxc.hu/.

Hybrid cultivars

The seed of a hybrid cultivar used for a commercial planting is produced by crossing two genetically different parents; therefore, the plants are heterozygous. New hybrid seed must be produced each year because the seed planted in a commercial field is not genetically the same as the seed harvested. There are multiple types of hybrids, including single-crosses, three-way crosses, and double crosses. They differ in the number of inbred lines that are used to produce the commercial seed. The plants in a single-cross hybrid are genetically the same or homogeneous, but the plants in a three-way or double-cross hybrid are genetically different or heterogeneous.

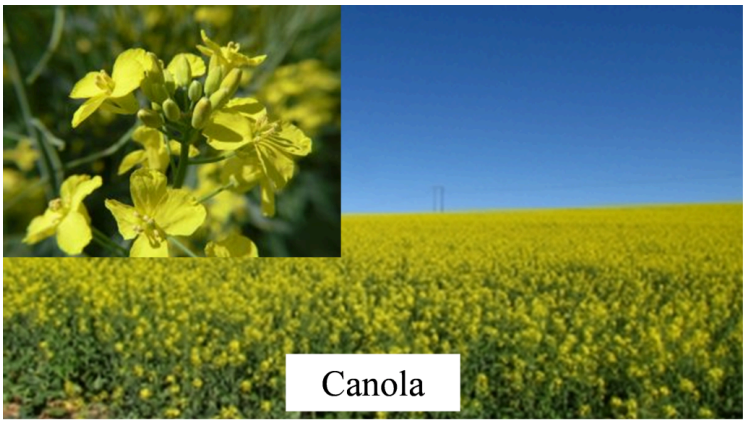


Figure 5. An example of a hybrid cultivar. Photo obtained from Stock.xchng at http://www.sxc.hu/.

Review Questions

1. Describe your experience with the breeding and the commercial production of any plant species.

2. Cultivars and germplasm lines of plant species can be registered in the *Journal of Plant Registrations* (JPR) and *Horticultural Science* (HortSci). For each of the four cultivar types listed below, use the 2010 or 2011 volumes of the journals to identify two plant species that utilize that cultivar type commercially, provide the name of a cultivar or an inbred line for a hybrid of your selected species, and the complete journal citation for the cultivar or inbred as used in the Reference section of JPR.

    a. Clonal cultivars

    b. Synthetic cultivars

    c. Pure-line cultivars

    d. Hybrid cultivars

3. Describe the homozygosity/heterozygosity and homogeneity/heterogeneity of the four cultivar types. Provide the rationale for your answer.

Sources of Parental Germplasm

Walter R. Fehr and Walter P. Suza

Readings:

- Chapter 10: Parent Selection [pdf], *Principles of Cultivar Development. Vol. 1: Theory and Technique*, by Walter R. Fehr (Access the full book)
- Chapter 11: Plant Introduction and Genetic Diversity [pdf], *Principles of Cultivar Development. Vol. 1: Theory and Technique*, by Walter R. Fehr

Introduction

The first step in the development of any cultivar is obtaining a segregating population in which to do selection. In most cases, the breeder develops the segregation population by crossing parents that have the potential of producing progeny with the traits desired in the new cultivar. The purpose of this lesson is to understand the sources of parent germplasm that a breeder may choose to use.

Sources of Parents

Cultivarsand elite breeding lines

The primary source of parent germplasm is **elite cultivars** and **experimental breeding lines**. Crosses among elite parents have the best chance of providing offspring that are superior to the parents and that will be useful as new cultivars.

The breeder of a plant species often will want to access elite cultivars and **breeding lines** from other breeders in private companies or public institutions. It is extremely important that a breeder have written permission to use germplasm developed by someone else and that the signed document be retained, in case any question is raised about the method by which the germplasm was received.

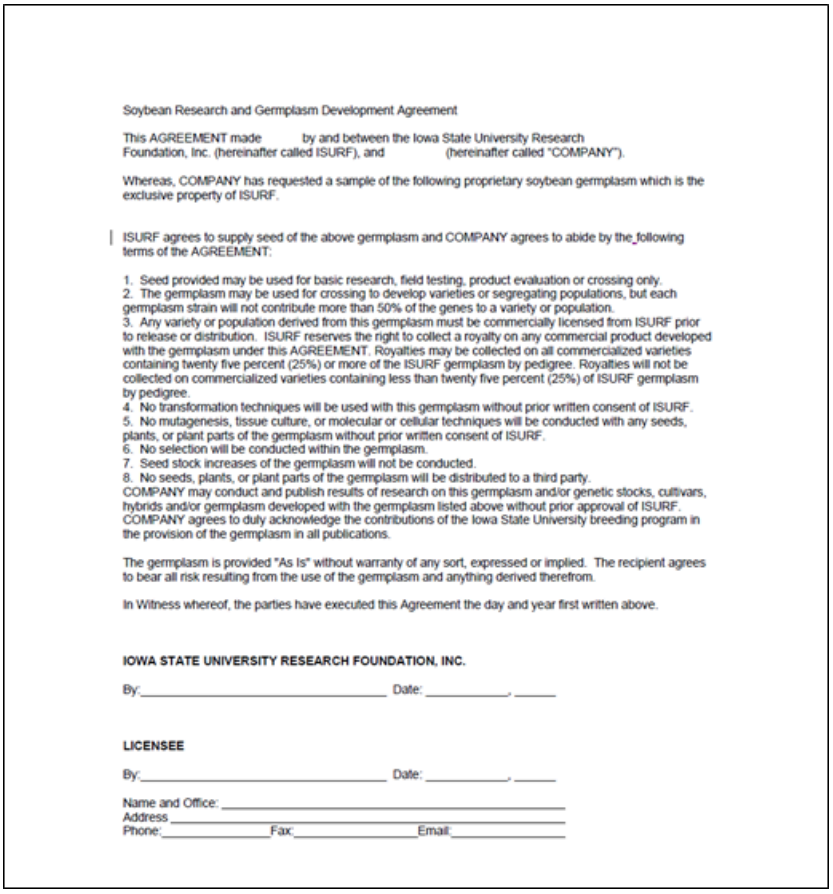
A verbal agreement is not sufficient when accessing parent germplasm from another breeder for several reasons.

1. The individual may not realize that they do not have the authority to make the verbal agreement.
2. The terms of a verbal agreement may be forgotten.

3. The individual with whom the verbal agreement is made may leave the company or institution.
4. The exchange of germplasm has become more restricted for both private and public institutions.

If an institution decides to restrict its exchange of parent germplasm, it may want to see a written document that verifies that germplasm previously received from it was properly obtained by another breeder. There are cases in which use of parent germplasm from another breeder without written permission has resulted in the loss of a job and legal awards of millions of dollars.

The terms of a written agreement vary among private and public institutions. The material transfer agreement at (Figure 1) from the Iowa State University Research Foundation at Ames, IA, is only one example of terms that may be involved in the exchange of parent germplasm.



Soybean Research and Germplasm Development Agreement

This AGREEMENT made by and between the Iowa State University Research Foundation, Inc. (hereinafter called ISURF), and (hereinafter called "COMPANY").

Whereas, COMPANY has requested a sample of the following proprietary soybean germplasm which is the exclusive property of ISURF.

ISURF agrees to supply seed of the above germplasm and COMPANY agrees to abide by the following terms of the AGREEMENT:

1. Seed provided may be used for basic research, field testing, product evaluation or crossing only.
2. The germplasm may be used for crossing to develop varieties or segregating populations, but each germplasm strain will not contribute more than 50% of the genes to a variety or population.
3. Any variety or population derived from this germplasm must be commercially licensed from ISURF prior to release or distribution. ISURF reserves the right to collect a royalty on any commercial product developed with the germplasm under this AGREEMENT. Royalties may be collected on all commercialized varieties containing twenty five percent (25%) or more of the ISURF germplasm by pedigree. Royalties will not be collected on commercialized varieties containing less than twenty five percent (25%) of ISURF germplasm by pedigree.
4. No transformation techniques will be used with this germplasm without prior written consent of ISURF.
5. No mutagenesis, tissue culture, or molecular or cellular techniques will be conducted with any seeds, plants, or plant parts of the germplasm without prior written consent of ISURF.
6. No selection will be conducted within the germplasm.
7. Seed stock increases of the germplasm will not be conducted.
8. No seeds, plants, or plant parts of the germplasm will be distributed to a third party.

COMPANY may conduct and publish results of research on this germplasm and/or genetic stocks, cultivars, hybrids and/or germplasm developed with the germplasm listed above without prior approval of ISURF. COMPANY agrees to duly acknowledge the contributions of the Iowa State University breeding program in the provision of the germplasm in all publications.

The germplasm is provided "As Is" without warranty of any sort, expressed or implied. The recipient agrees to bear all risk resulting from the use of the germplasm and anything derived therefrom.

In Witness whereof, the parties have executed this Agreement the day and year first written above.

IOWA STATE UNIVERSITY RESEARCH FOUNDATION, INC.

By:_____ Date: _____, _____

LICENSEE

By:_____ Date: _____, _____

Name and Office: _____
Address _____
Phone:_____Fax:_____Email:_____

Figure 1. Material transfer agreement from the Iowa State University Research Foundation.

Plant introductions

Plant introductions (PIs) in the United States are managed by the National Genetic Resources Program (NGRP). The NGRP is responsible for the acquisition, characterization, preservation, documentation, and

distribution of germplasm acquired from other countries or from breeding programs in the United States. The description of available plant germplasm and the method to acquire it can be found at www.ars-grin.gov.

Plant introductions have been a valuable source of novel genes for important traits for breeding programs. These include genes for insect resistance, disease resistance, seed quality, and many other traits. It common for breeders to evaluate plant introductions when searching for a trait that is not available in elite cultivars or breeding lines.

At the present time, plant introductions acquired through NGRP are available without a charge. Persons who obtain plant introductions are asked to provide the results of their evaluation to NGRP for the benefit of others who may be interested in the trait sometime in the future.

Review Questions

1. The purpose of this question is to help you understand the types of parents that are used in cultivar development. For each of the following cultivars listed in the January 2011 issue of the *Journal of Plant Registrations,* list the cultivar name, the species, and the parents crossed to form the population used for selection. Indicate whether the parents were cultivars, experimental lines, or plant introductions.

   To properly make answer this question, you must understand the symbolism that is used in the articles.

   a. A cultivar is denoted with apostrophes around the name, if the author does not specifically use the word “cultivar” before the name. For example, the cultivar Pella would be designated in an article as ‘Pella’, if the author did not say “cultivar Pella’. An experimental line, such as A38, would not have apostrophes around its name.

   b. Cultivars deposited in the NPGS are given a PI designation that is indicated in parentheses after the cultivar name, such as ‘Pella’ (PI123456). PIs that are not cultivars would not have a cultivar name associated with them.

   c. A parent designated as a brand can be considered a cultivar for the purposes of this assignment. The distinction between a cultivar and a brand will be discussed in Agron. 521.

   d. For some plant species, breeders designate the ancestry of a population, in addition to the immediate parents. The system is described in Crop Science 8: 405-406 (1968). The key point is that the immediate parents of a cross are located on either side of the single /. Those are the parents you should include in your answer.

      - Dan barley – JPR 5: 1-4 (2011):
      - Awesome Kentucky bluegrass – JPR 5: 5-10 (2011):
      - Au Red Ace red clover – JPR 5: 11-13 (2011):
      - Essex lentil – JPR 5: 19-21 (2011):

- Aloha seashore paspalum – JPR 5: 22-26 (2011):
- Bailey barley – JPR 5: 27-39 (2011):
- Intercross ryegrass – JPR 5: 40-44 (2011):
- Nelson annual ryegrass – JPR 5: 45-48 (2011):
- UA 4910 soybean – JPR 5: 49-53 (2011):
- Barlow wheat – JPR 5: 62-67 (2011):
- Merl wheat – JPR 5: 68-74 (2011):
- Snowglenn wheat – JPR 5: 81-86 (2011):
- Snowmass wheat – JPR 5: 87-90 (2011):
- SW049029104 wheat – JPR 5: 91-97 (2011):

2. Based on your survey in question 1, what percentage of the parents were cultivars, experimental lines, and plant introductions? What would explain the percentages that you obtained?

3. For a plant species of your choice, identify a plant introduction that you could acquire from the National Plant Germplasm System at www.ars-grin.gov. Provide the number of the PI that you have chosen and the rationale for your choice based on the traits you would like to obtain from the PI. Describe where the PI was acquired, where it is maintained in the United States, and how you would order it.

Population Formation by Hybridization

Walter R. Fehr and Walter P. Suza

Readings:

- Chapter 12: Population Formation by Hybridization [pdf], *Principles of Cultivar Development. Vol. 1: Theory and Technique*, by Walter R. Fehr (Access the full book)

Clonal, pure-line, and hybrid cultivars

Single crosses

The mating of two **elite parents** is the most common procedure for developing a population used in selection of clonal and pure-line cultivars, and inbred lines used in producing commercial hybrid cultivars. The genetic makeup of the hybrid seeds obtained from crossing two parents and the number of hybrid seeds desired from a **single cross** is markedly different for a single cross of two clonal parents compared with two pure-line or inbred parents.

The **clonal cultivars** and lines used in crosses are highly **heterozygous** and **homogeneous**. The hybrid individuals obtained from a single cross represent a segregating population that is used directly to begin selection for superior new clones. The number of hybrid seeds desired from a single cross is equal to the number of individuals that the breeder wants to evaluate as clones from the cross. If a breeder wants to evaluate 1,000 individuals from a single-cross population, at least 1,000 hybrid seeds would be produced. The number of plants of each parent used for crossing would not be important because they are genetically homogeneous.

The **pure lines** or inbred lines of hybrids used as parents are **homozygous** and homogeneous. Therefore, the hybrids obtained from a single cross are heterozygous and homogeneous. To develop a segregating F_2 population, the hybrid F_1 plants will be self-pollinated. The number of hybrid seeds obtained from a single cross is determined by the number of F_1 plants needed to produce the desired number of F_2 seed. If the breeder wants a total of 1,000 F_2 seeds and each F_1 plant produces an average of 100 seeds, only 10 hybrid plants would be sufficient. The number of hybrid seeds would be greater than 10 to account for hybrid seeds that do not germinate and the possibility of accidental self-pollinations. Assuming that the expected germination percentage is 80% and accidental self-pollinations are 10%, 14 hybrid seed would be desired (10 divided by

0.80 and 0.90). The number of plants of each parent used for crossing would not be important because they are homogeneous.

Three-way crosses and backcrosses

A three-way or backcross population is used whenever the expected frequency of progeny with the desired traits from a single-cross population may not adequate. A **three-way cross** also makes it possible to combine genes for desirable traits from more than two parents. A **backcross population** seldom is used in developing clonal cultivars because homozygosity will occur in the progeny that is negatively associated with performance of individuals. The genetic variability expected in a three-way population is greater than that for a backcross population. In a three-way population, two parents each contribute 25% of the genes and the third parent contributes 50%. In a backcross population, one parent contributes 25% of the genes and the recurrent parent contributes 75%.

The number of hybrids seeds that need to be obtained from the three-way cross or backcross is dependent on the number of gametes that the breeder wants to sample from the single-cross F_1 plants. Every gamete produced by the heterozygous, single-cross F_1 will be genetically different. The chance that a gamete with superior alleles from the two single-cross parents will unite with a gamete from the third parent or backcross parent is directly associated with the number of hybrid three-way or backcross seeds obtained.

Synthetic cultivars

The types of populations used for development of synthetic cultivars are more diverse than for clonal, pure-line, or hybrid cultivars. The following are some examples.

Cultivar per se

A synthetic **cultivar per se** can be used as a breeding population because of its inherent heterozygosity and heterogeneity. An example is the cultivar FreedomMR red clover [JPR 2: 205-207 (2008)]. The initial population used for selection was the cultivar, Freedom! The breeder used approximately 10,000 seeds of Freedom! to begin selection.

Single cross

When two synthetic cultivars are crossed, the parents *per se* would be highly heterozygous and **heterogeneous** and the hybrid seeds obtained from crossing the two parents would be heterozygous and heterogeneous. The number of hybrid seeds that need to be obtained would be equal to the number individuals from the population that the breeder wants to evaluate. As many different plants as possible of each parent would be used for crossing to capture the genetic heterogeneity of the parent. An example is the cultivar Intercross ryegrass [JPR 5: 40-44 (2011)]. It was selected from the single cross of 'Axcella' x 01-ARG. The F_1 seeds from the single cross were planted in the field and allowed to cross-pollinate. Selection began with the cross-pollinated seed.

Three-way cross

A three-way cross makes it possible to combine favorable genetic traits from three synthetic cultivars and lines. Nelson annual ryegrass is an example of a synthetic cultivar developed from a three-way cross [JPR 5: 45-48

(2011)]. The synthetic breeding lines TXR2000-T2 and TXR2002-T17 and the synthetic cultivar Jumbo were each planted in adjacent rows. Any plants that did not have good forage characteristics were removed and the remaining plants were allowed to cross-pollinate. The seed harvested from individual plants of the three parents was bulked to form the initial population for selection.

Polycross

In a polycross, selected plants (clones) are grown in an isolated nursery where cross-pollination occurs by wind or insect pollination. For the development of Warrior, a synthetic cultivar of indiangrass, the synthetic cultivar Oto was used as the base population (cycle 0) [JPR 4: 115-122 (2010)]. Every plant in Oto was genetically different. Twenty-nine selected clones from Oto were grown in two replications of an isolated polycross nursery and allowed to cross-pollinate by wind. Seed was harvested from individual clones and an equal amount from each was bulked to form the population for the next breeding cycle (cycle 2). In cycle 2, there were 742 clones evaluated of which 39 were selected and mated in a polycross to produce seed for cycle 3. In cycle 3, 875 clones were evaluated, of which 38 were selected and mated in a polycross. The seed harvested from the polycross of the 38 clones became the cultivar Warrior.

A **polycross** is commonly used by breeders of synthetic cultivars to cross selected individuals (clones) to each other. The number of clones involved in a polycross can vary from a few to more than 100. A polycross is used to (1) develop a segregating population from which new superior clones can be selected, (2) obtain half-sib seed from individual clones that can be used for evaluating their general combining ability, (3) obtain seed of an experimental synthetic for testing, and (4) obtain Syn.1 breeder seed of a new synthetic cultivar that will be released for commercial use.

Figure 1. The majority of polycrosses are done through natural open-pollination by wind or insects. However, there are a few crops for which a polycross can be done manually. These persons are making a polycross of selected alfalfa clones. Each pot in the circle is a different clones. Pollen is manually transferred among the clones. (Photo courtesy of Forage Genetics International).

Figure 2. For conducting a manual polycross, pollen is transferred among the parent clones by tripping their flowers with a folded piece of paper. Note the pollen on the tip of the paper. Intermating of the clones occurs when the pollen of different clones is mixed together as the flowers are tripped. The mixed pollen on the paper sticks to the stigma of a flower and fertilizes the eggs. (Photo courtesy of Forage Genetics International).

Figure 3. **(A)** A polycross for insect pollinated species can be done in cages. In this photo, selected alfalfa clones have been transplanted to cages where they will be intermated by bees. A bee trips the flowers in the process of collecting nectar. The pollen from the anthers sticks on body of the bee. As the bee travels from one flower to the next, a mixture of pollen from different plants is formed. Each time a flower is tripped, the pollen on its body is transferred to the stigma of the tripped flower and fertilizes its eggs. The resulting seed is a heterogeneous mixture of genotypes. **(B)** A polycross of alfalfa inside cages for pollination by bees. (Photos courtesy of Charles Brummer, Noble Foundation).

Figure 4. For plant species that are wind pollinated, polycrosses are generally made in the field in isolated plantings. In this field, the breeder has planted a polycross of tall fescue. The field was marked out by hand. The selected clones were transplanted to the field vegetatively. At the time of flowering, the pollen from the clones will be dispersed among the clones by wind, resulting in a heterogeneous mixture of seed on each plant. (Photo courtesy of Charles Brummer, Noble Foundation).

Figure 5. In this example, a rye border has been used to isolate the polycross of tall fescue clones. (Photo courtesy of Charles Brummer, Noble Foundation).

More complex populations

The goal of a more complex population is to combine together favorable alleles for multiple traits from a range of synthetic cultivars and lines. Au Red Ace red clover is an example of a cultivar selected from a complex population [JPR 5: 11-13 (2011)]. It was developed from five populations that together included the parentage of 18 commercial cultivars and five plant introductions.

Review Questions

1. Diagram the field layouts for mating 6 inbred parents of sorghum in a diallel for each of the following. Assume that 30 seeds are needed to plant each plot in a layout. Indicate the total number of plots and seeds needed for each layout.

    a. Unpaired parents with 3 dates of planting:

    b. Paired parents with 3 dates of planting:

    c. Semi-latin square with 1 date of planting:

    d. Bulk-entry with 1 date of planting:

2. Diagram the field layout for a partial diallel of 10 inbred parents of wheat by use of circular crossing in which each parent is mated to 4 other parents.

3. Diagram the field layouts for a polycross of 5 parent clones of bromegrass using the following designs. The number of replications for each design should be the same.

    a. Latin square

    b. Randomized complete-block

4. For each of the following cultivars in the January issue of the *Journal of Plant Registrations*, indicate the type of population that was formed for selection. Indicate if the hybrid seeds obtained by artificial hybridization were genetically homogeneous or heterogeneous.

    - Dan barley – JPR 5: 1-4 (2011)
    - Awesome Kentucky bluegrass – JPR 5: 5-10 (2011)
    - Au Red Ace red clover – JPR 5: 11-13 (2011)
    - Essex lentil – JPR 5: 19-21 (2011)
    - Aloha seashore paspalum – JPR 5: 22-26 (2011)
    - Bailey barley – JPR 5: 27-39 (2011)
    - UA 4910 soybean – JPR 5: 49-53 (2011)
    - Barlow wheat – JPR 5: 62-67 (2011)
    - Merl wheat – JPR 5: 68-74 (2011)

- Snowglenn wheat – JPR 5: 81-86 (2011)
- Snowmass wheat – JPR 5: 87-90 (2011)
- SW049029104 wheat – JPR 5: 91-97 (2011)

5. What determined if the hybrid seeds obtained by artificial hybridization were genetically homogeneous or heterogeneous?

Techniques for Artificial Hybridization

Walter R. Fehr and Walter P. Suza

Readings:

- Chapter 13: Techniques for Artificial Hybridization [pdf], *Principles of Cultivar Development. Vol. 1: Theory and Technique*, by Walter R. Fehr (Access the full book)
- Chapter 14: Interspecific Hybridization [pdf], *Principles of Cultivar Development. Vol. 1: Theory and Technique*, by Walter R. Fehr
- Chapter 1: Environmental Effects on Flowering [pdf], *Hybridization of Crop Plants*, Walter R. Fehr and Henry H. Hadley (Eds.) (Access the full book)

Introduction

The first step in cultivar development is obtaining segregating populations from which superior individuals can be selected. Chapter 2 covered the selection of parents that could be used for hybridization to form a segregating population. In Chapter 3, the various types of segregating populations and the field layouts for mating parents were discussed. The purpose of this chapter is to understand the procedures used to obtain hybrid seed from the mating of selected parents for a breeding program. The production of hybrid seed for developing segregating populations is not done in the same manner as the production of seed of hybrid cultivars for sale to end users. The production of commercial quantities of seed of hybrid cultivars will be covered in *Principles of Cultivar Development*.

Artificial hybridization requires knowledge of the species mode of reproduction and the conditions necessary to obtain flowers of the parents at the same time. You will want to review lesson 1 of Agron. 527, and the reading assignment for this lesson to understand the types of flowers and modes of reproduction.

From the perspective of artificial hybridization, the three basic types of flowers are perfect, monoecious, and dioecious. **Perfect flowers** contain both the male and female organs. Species with **monoecious flowers** have the female and male organs on different parts of the plant (Figure 1). Species with **dioecious flowers** have the female and male organs on different plants, for example, buffalograss: (YouTube)

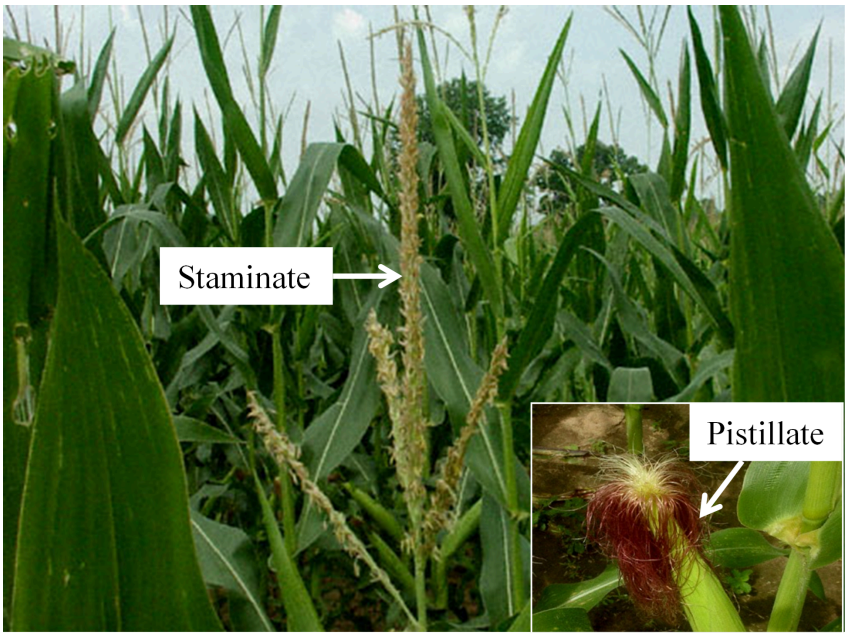


Figure 1. Maize is a monoecious species with both staminate (male) and pistillate (female) flowers on the same plant. Photo courtesy of Shui-zhang Fei, Iowa State University.

Hybridization of species with perfect flowers

The mating of parents that have perfect flowers can be done with or without manual labor. Manual crossing is done when the floral parts are of adequate size, control of the female and male parents of the hybrid seed is desired, and plants that result from self-pollinated seed can be readily distinguished from hybrid plants. Examples of species that routinely used manual crossing are barley, common bean, field bean, soybean, oat, triticale, canola, and wheat. Figures 2 to 12 illustrate manual crossing in soybean and provide important information about flower anatomical features to be take into consideration in this process.

Figure 2. The soybean has a small perfect flower. Some persons find it helpful to wear magnifying lenses to enlarge the reproductive organs. Photo courtesy of Walter R. Fehr, Iowa State University.

Figure 3. Soybeans in Iowa begin to flower in the field when they are less than half their final height. Photo courtesy of Walter R. Fehr, Iowa State University.

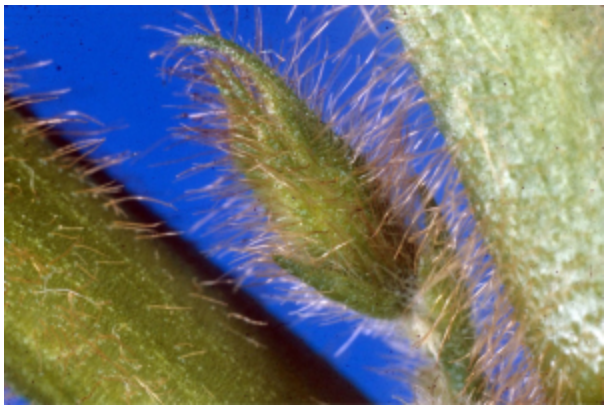


Figure 4. This flower is at the stage when the female is receptive to pollen, but the anthers are not mature enough to shed pollen. A flower of this stage on the female parent will be used for crossing. All of the flowers at the node that have fully exposed petals and the young buds that will become flowers are removed with a tweezers. This reduces competition for nutrients between the flowers used for crossing and other flowers at the node. Photo courtesy of Walter R. Fehr, Iowa State University.

Figure 5. The sepals surrounding the petals are removed with a tweezers. The petals of soybean flowers are primarily white (recessive) or purple (dominant). If one parent of a cross is white and the other is purple, the white-flowered plant will be used as the female so that accidental self-pollinations can be readily detected when the hybrid plants are grown. Photo courtesy of Walter R. Fehr, Iowa State University.

Figure 6. The petals of the flower are removed to expose the reproductive organs. The scar that marks the place where the sepals were removed will be used to differentiate at harvest pods from flowers that were used for artificial hybridization and pods resulting from natural self-pollination that have their full sepals. Photo courtesy of Walter R. Fehr, Iowa State University.

Figure 7. The female reproductive organ is surrounded by 10 anthers. This flower is ideal to use for crossing because the anthers are not mature enough to shed pollen. As the anthers mature, the filaments increase in length so that the anthers surround the stigma when they shed pollen. Emasculation of the flower is not necessary because if the exposed stigma is not pollinated, it will dry up and be unreceptive by the time the anthers of the flower are mature enough to shed pollen. Photo obtained from Fehr, W.R. 1980. Soybean. p. 593. In W.R. Fehr and H.H. Hadley (eds.) *Hybridization of crop plants*. The American Society of Agronomy and the Crop Science Society of America, Wisconsin.

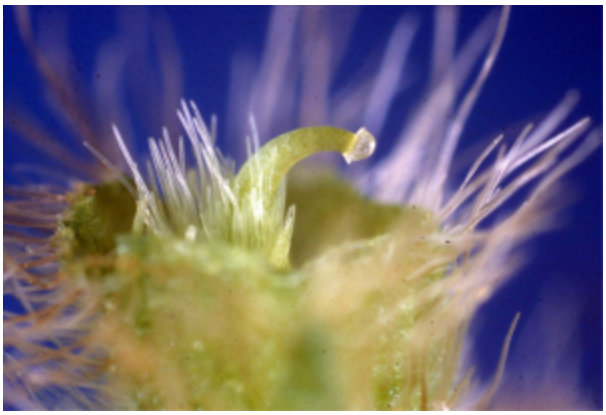


Figure 8. The stigma at the end of the style looks like a drop of water. Photo courtesy of Walter R. Fehr, Iowa State University.

Figure 9. When the petals of a flower on the male parent are fully exposed, the anthers are ready to shed pollen. Photo obtained from Fehr, W.R. 1980. Soybean. p. 593. In W.R. Fehr and H.H. Hadley (eds.) *Hybridization of crop plants*. The American Society of Agronomy and the Crop Science Society of America, Wisconsin.

Figure 10. A tweezer is used to remove the male and female reproductive organs from a flower of the male parent. Note that the anthers are surrounding the stigma. Photo courtesy of Walter R. Fehr, Iowa State University.

Figure 11. The anthers are gently touched on the stigma so that the pollen is released from the anther. One male flower can be used to pollinate up to three female flowers, if pollen production is good. Photo courtesy of Walter R. Fehr, Iowa State University.

Figure 12. About seven days after pollination, a pod will be visible if crossing was successful. Only about 50% of the female flowers successfully produce a pod and there are only about two seeds in a pod. This means that on the average only one hybrid seed is obtained from the female flowers used for crossing. The dried stigma at the tip of the pod will remain when the pod is mature. The seed scar from removal of the sepals is clearly visible. Photo obtained from Fehr, W.R. 1980. Soybean. p. 593. In W.R. Fehr and H.H. Hadley (eds.) *Hybridization of crop plants*. The American Society of Agronomy and the Crop Science Society of America, Wisconsin.

Crossing of parents without manual labor is done when floral parts are extremely small, self-pollination is minimized or eliminated through self-incompatibility or male sterility, cross-pollination is common by wind or insects, and individuals that are the result of self-pollination can be readily distinguished from hybrid individuals. Manual labor is not used for species that routinely use a polycross to mate multiple parents to form a population for selection, as discussed in lesson 4. Examples of species that commonly use a polycross include, alfalfa, bromegrass, orchardgrass, red clover, and switchgrass,

Sugarcane is an example of a species with perfect flowers for which manual labor generally is not used for crossing. The inflorescence of sugarcane, also called a flowering arrow or tassel, consists of thousands of both perfect and imperfect flowers. The imperfect flowers are a result of abortion of either the pistils, but to a greater extent of the stamens. Parental genotypes of sugarcane have a broad variation in fertility and seed production due to male sterility and self-incompatibility.

Parental clones generally are selected before flowering, potted, and maintained in an isolated facility designated for crossing. Manual **emasculations** generally are not done because too many small flowers are produced per inflorescence and it takes time for the whole arrow to complete flowering. For cross-pollination, breeders commonly select female parents that are largely male sterile. Pollination may be done by placing the arrow of the male parent above the arrow of the female parent so that pollen can fall on the female flowers. Alternatively, pollen may be collected from the flowers of the male parent and dusted over the arrow of the female clone.

If the female parent is male-sterile, only hybrid seed will be obtained. A cross to a male-fertile female will result in the production of both selfed and hybrid seed. Individuals resulting from self-pollination generally will be eliminated during selection because they will not perform well due to inbreeding depression. When available, molecular markers also can be used to distinguish selfed and hybrid individuals.

Hybridization of monoecious species

Watermelon is an example of a **monoecious** species (Figure 13A) for which manual labor is used for artificial hybridization. In commercial production, watermelon is naturally pollinated by insects. Prior to manual pollination, female flowers may be protected from insect pollinators by using paper clips to restrict buds from opening, covering with foam cups, or small screen cages and nets. The male flowers also may be protected by the same methods used to protect female flowers to prevent contamination by pollen from other plants. It may be unnecessary to protect the flowers of plants grown in greenhouses equipped with structures that exclude insects. The process of hand pollination involves the harvesting of male flowers, opening their petals, and brushing the male flower against the female flower as shown in Figure 14. After pollination, female flowers should remain protected for one day to prevent accidental pollination.

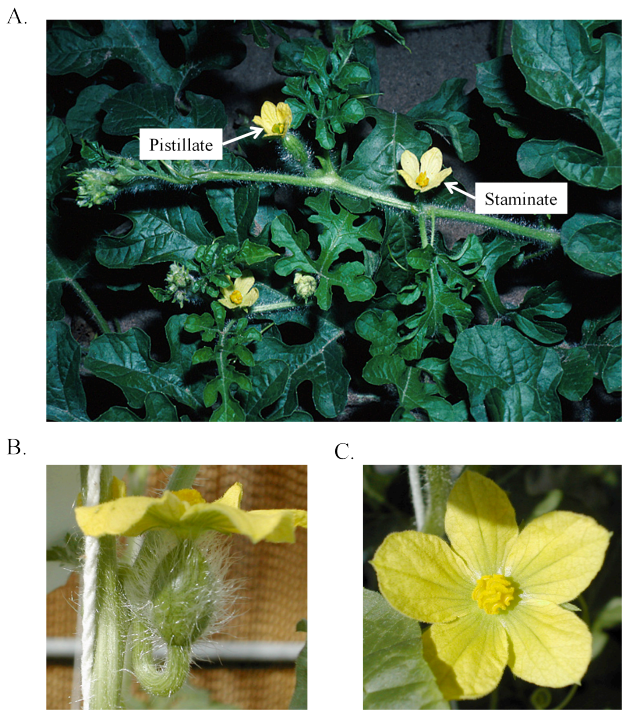


Figure 13. Watermelon is a monoecious species with both (B) pistillate and (C) staminate flowers on the same plant. Photo in 4A courtesy of Shui-zhang Fei, Iowa State University. Photos in 4B and 4C from watermelon breeding handbook, by Gabriele Gusmini at https://cucurbitbreeding.wordpress.ncsu.edu/

Figure 14. Hand pollination of watermelon involves brushing the male flower against an open female flower. Photo from watermelon breeding handbook, by Gabriele Gusmini at https://cucurbitbreeding.wordpress.ncsu.edu/

Hybridization of dioecious species

Pistachio is an example of a species for which manual labor is used for **artificial hybridization**. The following

description is based on the article by authors in the Journal of Plant Sciences describing the effects of artificial pollination on pistachio fruit cropping [J. Plant Sci. 2:228-232 (2007)].

The pistachio is naturally wind-pollinated in commercial production. For artificial hybridization female flowers (figure 15A) are normally protected using paper bags before they are fully developed. Male trees normally bloom earlier than when the female flowers are fully mature and ready for pollination; therefore, pollen is collected from the male flowers (figure 15B) and stored at $4^{o}C$ until the female flowers are ready for pollination. Application of pollen to the female flowers is done using a brush. The pollinated flowers are covered to prevent accidental pollination.

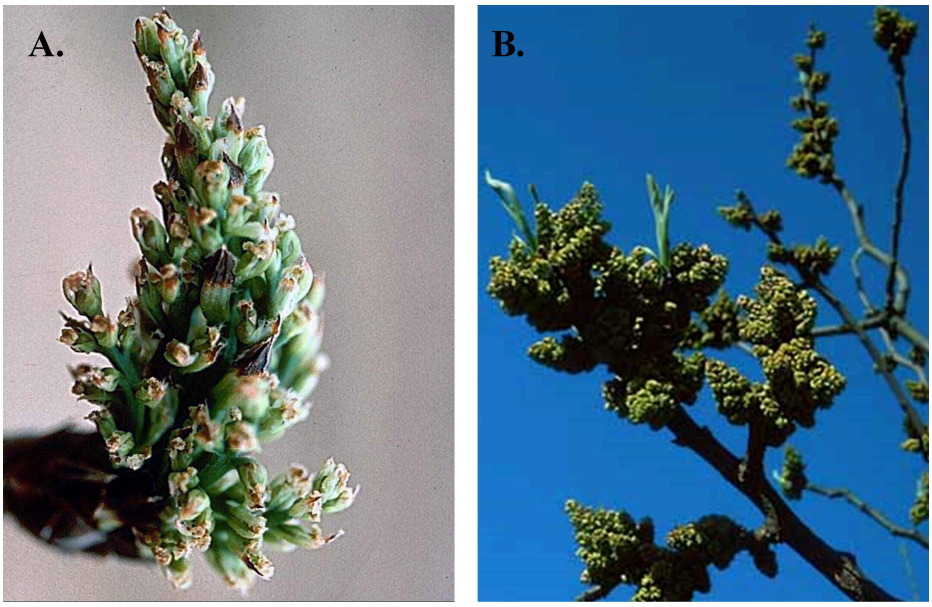


Figure 15. Pistachio plants produce either (A) female or (B) male flowers.

Review Questions

In this assignment, you will use the chapters for the following species from the book Hybridization of Crop Plants: rice, maize, and hop. For each species, briefly describe the following:

Question	Rice	Maize	Hop
(1) Flower structure and distribution on the plant.			
(2) Environmental conditions that control flowering and at least one strategy to get parents to flower at the same time.			

Question	Rice	Maize	Hop
(3) Preparation of the female flower for pollination.			
(4) Collection of male pollen.			
(5) Techniques for pollination.			
(6) Protection of the pollinated flower from contamination.			
(7) Labeling of the pollinated flower.			
(8) Method of harvesting hybrid seed. This refers to hybrid seed obtained by crossing of selected parents for population development, not commercial hybrid seed production.			
(9) Number of seeds generally obtained from one pollination.			

Mutation Breeding

Walter R. Fehr and Walter P. Suza

Readings:

- Chapter 20: Mutation Breeding [pdf], *Principles of Cultivar Development. Vol. 1: Theory and Technique*, by Walter R. Fehr (Access the full book)

Introduction

Over the centuries, genetic improvement of plants has been achieved by utilization of the genetic variability from naturally occurring mutations. Although naturally occurring mutations can still be a valuable source of novel genes, there are instances in which none of the available germplasm of a plant species has a trait needed by a breeder. For example, soybean breeders were interested in modifying soybean oil to improve its shelf-life. None of the cultivars or the thousands of plant introductions had the necessary trait. Through the use of mutagenesis, genes were developed that made it possible to develop cultivars with the improved oil characteristics.

The text provides basic information on the factors that a breeder must consider in designing and conducting a mutagenesis program. The following discussion is intended to provide examples of how these principles were applied in creating novel genes. The text provided in the shaded boxes has been provided to clarify and expand on general principles of mutagenesis and is not part of the text of those articles.

Example 1

The information provided below for the development the rice cultivar Calrose 76 was obtained from Crop Sci. 16:631-635 (1976), Crop Sci. 17:978 (1977) and Induced Plant Mutations in the Genomics Era:44-47 (2009).

Step 1.

The cultivar Calrose was selected for irradiation.

The advantage of using an elite cultivar for mutagenesis is that it may be possible to identify a mutant that is suitable for release as a new cultivar without additional breeding, as was the case for Calrose 76. However, a plant with the desired mutation may not be suitable for release as a cultivar because it may contain undesirable mutations for other traits of importance. In those cases, the mutant allele is incorporated into elite germplasm by hybridization and selection.

Step 2.

There were 2,050 seeds of Calrose treated with each of five dosages of the physical mutagen cobalt-60: 0, 15, 20, and 25 kR.

The use of five dosages increased the chance of finding one dosage that would result in an adequate mutation frequency. The use of a control treatment made it possible to assess how each of the dosages influenced germination and the frequency of mutation.

Step 3.

Before treatment, the seeds were brought to 14% moisture.

Adjustment of moisture would be considered a pretreatment. It is assumed that the breeder had some prior knowledge that this step would be useful when using cobalt-60 radiation.

Step 4.

The M_1 seeds were planted in the field. Although rice is self-pollinated, the M_1 plants were isolated by 11 m from untreated rice genotypes to prevent outcrossing.

If a M_1 plant had a recessive mutation, its M_2 progeny would be expected to segregate in a 1:2:1 ratio. If outcrossing occurred and gametes with the wild-type allele from the untreated genotypes were responsible for fertilization, instead of those from the M_1 plant itself, the frequency of homozygous mutant M_2 progeny from a heterozygous M_1 plant with a recessive mutation would be reduced to less than the expected ¼ frequency. This would make it more difficult to find M_2 individuals with the desired mutation.

Step 5.

There were 200 individual plants harvested from each treatment and approximately 1,400 plants were harvested in bulk.

By harvesting individual plants, the breeder could have grown progeny from each. If an M_1 plant was heterozygous for a mutant allele, recovery of a homozygous mutant M_2 plant in the $M_{1:2}$ line would be expected if a sufficient number of progeny were grown. To be 95% sure that at least one M_2 plant from a heterozygous M_1 plant would be homozygous for the mutation, 11 individuals would have to be evaluated in each $M_{1:2}$ line. See page 367 of chapter 28 for an explanation of how this calculation was made. For 200 M_1 plants and 11 progeny from each, a total of 2,200 M_2 individuals would have to be evaluated.

The breeder also used a second alternative for managing the population, which was to harvest about 1,400 M_1 plants from each dosage in bulk. This made it possible to evaluate progeny from more M_1 plants, but reduced the number of M_2 progeny that could be evaluated from each. If 2,200 M_2 progeny were evaluated from the bulk, an average of only two M_2 progeny from each of the 1,400 M1 plants would be tested from each. This illustrates an important choice that the breeder must make in harvesting and testing M_2 progeny from a mutagenized population ie. testing more progeny of fewer M_1 plants or fewer progeny from more M_1 plants.

Step 6.

At maturity, short statured M_2 plants were harvested individually. Undesirable plants, including haploids, triploids, and many semi-steriles were discarded.

It is common to find abnormalities in plants from mutagenized seed, particularly when physical mutagens such as gamma radiation are used.

Step 7.

The progeny of M_2 plants were grown as $M_{2:3}$ lines to identify those that were homozygous for short stature. Eleven of the lines were selected for further evaluation.

Progeny testing is essential to confirm that an individual plant has a mutant allele and whether the allele is stable from one generation to the next.

Step 8.

The selected lines were evaluated for yield in subsequent generations. One of the experimental lines, D7, became the cultivar Calrose 76.

The breeder carried out another important step by conducting a genetic study to determine the inheritance of the short statured trait in Calrose 76. This information was important for using the allele effectively in a rice breeding program. Short stature in Calrose 76 was found to be controlled by a single recessive allele that was designated *sd1*.

Example 2

Information found in Plant Breeding 129:412-416 (2010) describes the treatment of vegetative tissue of St. Augustinegrass by gamma irradiation to induce semi-dwarf mutants to be used as breeding material for cultivar improvement.

Step 1.

The clonal cultivar Raleigh was selected for irradiation. Raleigh had superior cold tolerance and was widely used as a home lawn in the southern U.S.

Step 2.

About 140 single node cuttings of uniform size from randomly sampled stolons were treated with cobalt-60. Irradiation treatment was conducted at a facility in the Nuclear Engineering Department at North Carolina State University. Dosages of 0, 50, 60, 70, 80, 90 and 100 Gy were used (1 Gy = 0.1 kR). For each dosage, 20 cuttings were treated. The single node cuttings used as the control were maintained in moist conditions to minimize dehydration. Dosage effect on callus tissue also was determined with 0, 25, 50, 100, and 200 Gy.

> The importance of treating cuttings of uniform size was to ensure that radiation penetration was uniform for all materials treated. The inclusion of calli in this research was to test if the tissue is amenable to gamma irradiation. It was unknown whether calli could be used as starting material for mutagenesis in grass species.

Step 3.

The doses 50 to 70 Gy gave reasonably high survival rate for the cuttings. Based on this information, an additional 250 cuttings were treated for each of the 50 or 70 Gy dosages. The success of recovering plants from radiation-treated callus was higher for doses 25 and 50 Gy.

Step 4.

Plants obtained from radiation-treated node cuttings or calli were screened for freezing tolerance. Those that survived the cold treatment were screened for morphological changes.

> It was important to screen for cold tolerance because any useful morphological mutants would need to be acceptable for the trait.

Step 5.

A total of 13 semi-dwarf mutants with tolerance to cold were obtained.

Example 3

An example of using chemical mutagenesis to develop herbicide tolerance in barley is described in [PNAS 108:8909-8913 (2011)].

Step 1.

The barley cultivar Bob was selected for mutagenesis with sodium azide to develop tolerance to imidazolinone herbicides.

Step 2.

Mutagenesis was done by treating about 500 grams of seed with 1mM sodium azide for 2 h.

Step 3. Before treatment, seed were pre-soaked at 0 ^{o}C for 16 h and subsequently at 20 ^{o}C for 8 h.

Step 4.

The M_1 seed was planted in the field in isolation. The M_2 seed was harvested in bulk.

Step 5.

About 2 million M_2 plants were grown in a greenhouse where they were screened for resistance to the imidazolinone herbicide. The screening procedure involved placing 250 seeds in a Petri dish and soaking them with a 1,120.3 µM solution of imazethapyr for 48 h. The imazethapyr-treated seeds were subsequently planted in a commercial potting mix in flats that held 1000 seedlings. The seedlings were grown in a greenhouse under 16 h of light (22 ^{o}C) and 8 h of dark (16 ^{o}C). After four weeks in the greenhouse, seedlings were visually inspected for tolerance to the herbicide. Herbicide tolerant seedlings were transferred to pots and maintained under the same greenhouse conditions.

Step 6.

M_2 plants showing tolerance to the herbicide were allowed to self-pollinate and each plant was harvested individually. About 5 to10 M_3 progeny were grown from each M_2 plant for seed increase. The M_4 seed from each $M_{2:3}$ line was harvested and each $M_{2:4}$ line was evaluate for tolerance to the herbicide. Out of the 2 million M_2 seedlings initially screened, only one line was found to be homogeneous for herbicide tolerance.

Step 7.

Herbicide tolerance was associated with a slight delay in seed germination. Thus, the new mutant line was backcrossed to Bob.

It is common that mutant lines have undesirable traits that preclude their use directly as a cultivar. The breeder must attempt to eliminate the negative traits by conventional hybridization and selection.

Table 1. Examples of germplasm developed with alleles obtained by mutagenesis.

Crop	Mutant Gerplasm	Mutation Method	New Trait	Reference
Rice	Calrose 76	Gamma rays	Short stature	Crop Sci. 17: 978 (1977)
Wheat	FS4	Sodium azide	Herbicide tolerance	Plant Physiol. 100: 882-886 (1992)
Soybean	A29	Ethyl methanesulfonate	Reduced linolenate	US Patent No. 6 133 509. October 17, 2000
	C1726	Ethyl methanesulfonate	Reduced palmitate	Crop Sci. 30: 240 (1990)
	A22	*N*-nitroso-*N*-methylurea	Reduced palmitate	Crop Sci. 31: 88-89 (1991)
Oats	Alamo-X	X-rays	Disease resistance	Crop Sci. 2: 531 (1962)
Bermudagrass	TifEagle	Gamma rays	Better turf quality when mowed	Crop Sci. 39: 1258 (1999)
	TifWay II	Gamma rays	Nematode and cold tolerance, faster growth	Crop Sci. 25: 364 (1985)
St. Augustine grass	TXSA 8202	Gamma rays	Disease resistance	Crop Sci. 25: 371 (1985)
	TXSA 8218	Gamma rays	Disease resistance	Crop Sci. 25: 371 (1985)

Figure 1. Calrose 76 was the first semi-dwarf rice cultivar in the USA produced by gamma irradiation. The CS-M3 cultivar without the mutant sd1 allele is 75% taller than Calrose 76. Photographs are from Rutger JN (2009) in: Q.Y. Shu (ed.) Induced Plant Mutations in the Genomics Era. FAO, Rome, 2009, 44-47.

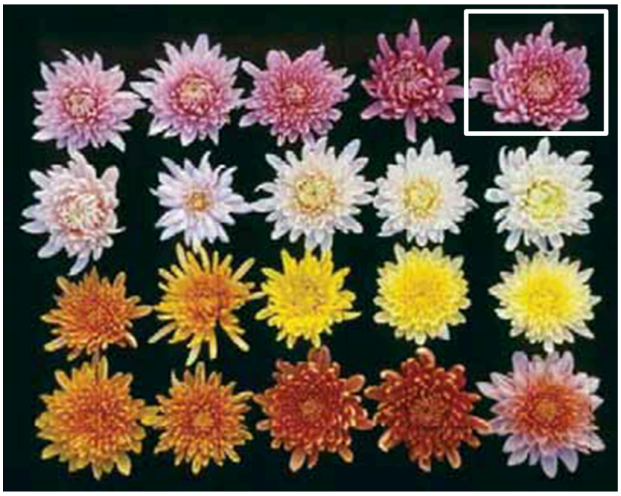


Figure 2. Flower color mutant lines of Chrysanthemum produced by gamma field irradiation of the cultivar Taihei. A flower of the original cultivar is enclosed in a box in the upper right hand corner. Adapted from Nagatomi S and Degi K (2009) in: Q.Y. Shu (ed.) Induced Plant Mutations in the Genomics Era. FAO, Rome, 2009, 258-261

Figure 3. Gamma irradiation field (Gamma Field) at the Institute of Radiation Breeding in Japan. The mutant lines of Chrysanthemum shown in Figure 2 were derived from this method of mutagenesis. Photograph is from Nakagawa H (2009) in: Q.Y. Shu (ed.) Induced Plant Mutations in the Genomics Era. FAO, Rome, 2009, 48-54.

Review questions

You are a maize breeder who has the responsibility to develop an inbred line that has 80% oleic acid in the oil. Your first step was to evaluate the oleic acid content of the elite inbreds used in hybrids and inbreds available in the National Plant Germplasm System. The range in oleic acid among those inbred lines was from 16 to 48%. Consequently, you have decided to use mutagenesis in an attempt to develop a mutant allele that would increase oleic acid to 80%.

Design a mutagenesis program that could be used in an attempt to meet your objective. Provide specific information for each of the following and the rationale for your choice.

a. Name of the mutagen you would use for the program and the reason for your choice.

b. What characteristics would you consider in selecting the parent inbreds for treatment?

c. How many inbred lines would you use if you can treat at total of 20,000 M_0 seeds?

d. Outline the steps in your treatment procedure beginning with the M_0 seeds and ending when you have the M_1 seeds ready to plant. Be sure to include any pre-treatment steps.

e. How would you manage the M_1 plants in terms of pollination and harvest?

f. You can evaluate 40,000 samples for oleic acid content by gas chromatography. A sample can be an individual seed from a plant, but you must destroy the seed in the process. Alternatively, you can use as a sample a bulk of five seeds from a plant. Describe how you would evaluate the M_2 seeds you harvested in the preceding question e.

g. What would you grow in the M_3 generation and how would you manage the pollination and harvest?

h. How would you confirm that an individual with 80% oleic acid is homozygous and homogeneous for a mutant allele?

Novel Traits from Molecular Genetics and Biotechnology

Walter P. Suza and Walter R. Fehr

Introduction

Research in molecular genetics and biotechnology is providing new tools for selecting genes of interest and for generating new genes of economic importance. The use of these new technologies for cultivar development is the subject of this chapter.

Molecular markers

A **molecular marker** is part of the DNA of a plant associated with the DNA of a gene of interest. The DNA of a molecular marker may be linked (linked marker) to a gene of interest or part of the gene itself (perfect, direct, or functional marker).

The use of a molecular marker is an alternative to the use of phenotypic selection for selecting a gene of interest. For example, the dominant *Rag1* allele provides partial resistance to an aphid in soybean. A molecular marker is available to identify plants that are homozygous for the desired allele. If the marker was not available, a breeder would have to infect plants with aphid to identify those with the *Rag1* allele. Plants that had a reduced number of aphids could be homozygous or heterozygous for the allele or could be plants without the allele that accidentally escaped aphid infection. Consequently, phenotypic evaluation would be less reliable and more expensive than the use of a molecular marker.

DNA markers can be useful in species that have long life cycles (e.g. trees) or if a trait is expressed only at later stages of development. For example, in asparagus [Euphytica. 94:329–333 (1997)] and gingko [Euphytica. 169:49-55 (2009)], identification of an individuals' sex is desirable at early stages, but may not be expressed until plants are more than two years old (asparagus) or have reached sexual maturity (gingko).

Breeders must decide on a case-by-case basis if the use of a molecular marker will be more efficient and cost effective than the use of phenotypic selection. For example, transgenes that provide herbicide resistance are widely used in some plant species, such as soybeans and maize. Molecular markers generally are not used for selection of plants with a herbicide resistance gene because it is less expensive to spray plants with the herbicide and kill those without the gene.

Common types of markers

The two most common types of markers currently used are a simple sequence repeat (SSR) and a single nucleotide polymorphism (SNP). Both types are used as linked markers. SNPs also can be used as a direct marker for some alleles.

Simple Sequence Repeat (SSR)

SSRs are pieces of DNA containing repeated nucleotide sequences. The repeated nucleotide sequences in plants can be two bases, such as AT, AG or TC [Trends in Plant Sci.1:215-222 (1996)] or three bases, such as ATT [Plant J. 3:175-182 (1993)]. To effectively use an SSR as a marker, two criteria need to be met. (1). The SSR must be tightly linked to the gene of interest so that they are not separated by a crossover during meiosis. Ideally, there would be a tightly linked SSR on both sides of the gene so that it is possible to determine if a crossover did occur. (2). The SSR must be **polymorphic** in the parents used to form the population from which individuals are being evaluated. Individuals with the desired allele must have one SSR sequence and those with the other allele must have a different SSR sequence.

Single nucleotide polymorphism (SNP)

SNPs are single nucleotide differences in the DNA of two individuals. For example, one individual may have an adenine (A) and another individual may have a guanine (G) at the same location in the DNA sequence. If a SNP is used as a linked marker, it must be tightly linked to the gene of interest and polymorphic in the parents of a population. A SNP within the gene that controls a trait is ideal because there is no possibility of a crossover between the SNP and the allele of interest.

Detection of molecular markers

To detect molecular markers, thousands of copies of the DNA region that differentiates individuals that do or do not have the allele of interest are made by PCR. The process of making the copies is referred to as amplification. The method invented by Kary Mullis in the mid-1980s is a way of amplifying minute quantities of DNA [Methods in Molecular Biol. 226:3-6 (2003)]. The basic materials used to conduct a PCR reaction are the following.

1. *DNA from the individual to be evaluated.* This could be obtained from seed or vegetative tissue, particularly young leaf tissue. Figure 1 is an example of the DNA sequence of a region that will be amplified.

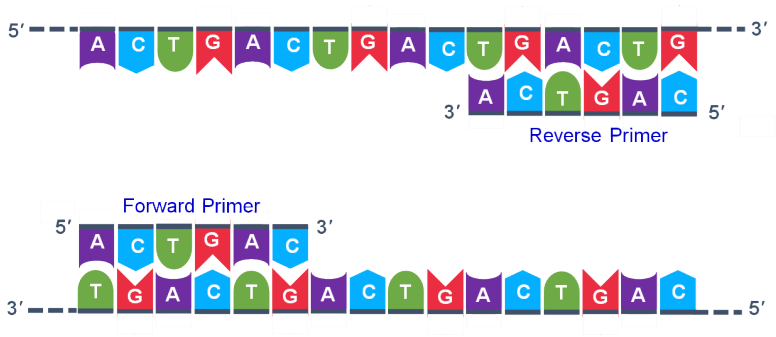


Figure 1. Primers for Taq polymerase. Primer 1 and 2 are complementary to their target sequence in the two DNA strands. Arrows indicate the direction of the new DNA strand synthesized by Taq polymerase as guided by the primers.

2. *The DNA polymerase enzyme called Taq polymerase*. It originally was isolated from a bacterium called *Thermus aquaticus* from which the name of the enzyme was derived. Taq polymerase has a temperature optimum of 72^{o}C and is stable even at 95^{o}C.
3. *DNA primers that match the sequence on either side of the region to be amplified*. The primers are made in machines called DNA synthesizers. Figure 1 shows the primers that will be used to amplify the region of interest by the Taq polymerase.
4. *Deoxynucleoside triphosphates (dNTPs) that contain the four nucleotides (A.G.C.T) that are used to make the new strands of DNA.*
5. *A thermo cycler that controls the temperature for the PCR reaction.*

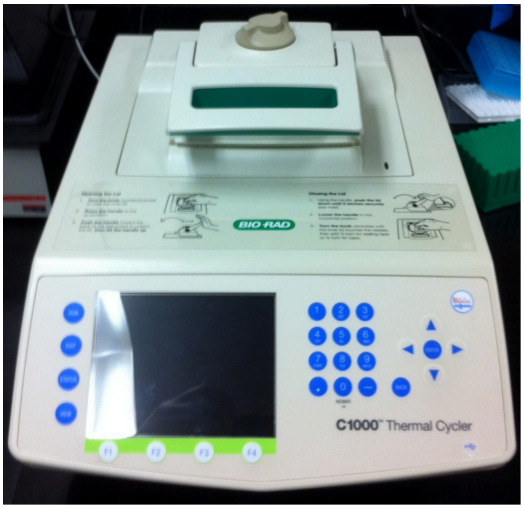


Figure 2. The thermocycler is used in the laboratory to amplify DNA by the PCR method.

The PCR reaction involves three basic steps. Repetitions of the three steps are referred to as cycles:

- **Step 1. Denaturation** – heating the DNA to about 95^{o}C causes the two strands to come apart
- **Step 2. Annealing** – primers bind to complementary regions of the two separated strands
- **Step 3. Extension** – the polymerase uses primers and dNTPs to synthesize new DNA strands

The following are two links that will provide you with an illustration of how PCR works. The first link is an exercise that will help you understand the way in which the four DNA bases in the PCR mixture are matched with their counterpart during the reaction and how the strands are copied during the reaction:

- Laboratory Protocols[1]

The second link is an animation of the process.

- PCR Animation Video[2]

The polymorphic SSR marker (Figure 3) can be evaluated with gel **electrophoresis**. Prior to electrophoresis, the DNA is loaded on a gel that is placed on a box in which there is a current running from one end to the other (Figure 4). DNA is negatively charged and will migrate through the gel toward the positive pole.

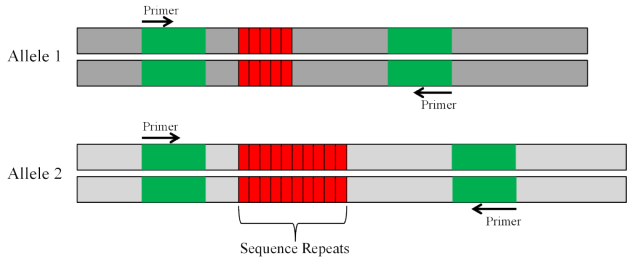


Figure 3. The SSR alleles 1 and 2 differ by the number of repeating DNA units. PCR primers are designed to amplify the region containing the DNA repeats. The difference in number of repeats in the two alleles causes a difference in the length of their PCR product, which can be distinguished when the PCR products are analyzed on a gel. An SSR marker should be closely linked to an allele for an important trait.

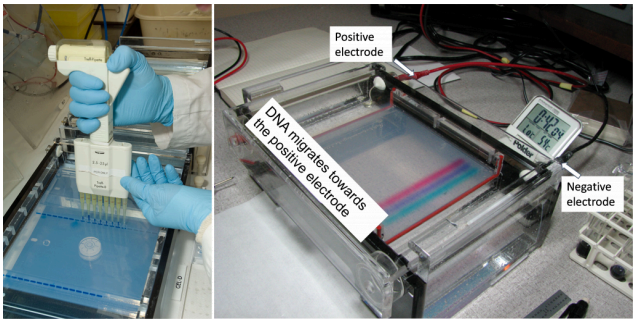


Figure 4. Analysis of PCR products by gel electrophoresis. The DNA sample is placed in the gel at the negative pole from which it will migrate to the positive pole. (Picture taken by Mike Zeller, Office of Biotechnology, Iowa State University).

During electrophoresis, longer fragments of DNA travel through the gel slower than smaller fragments, which makes it possible to differentiate the size of the fragments by the position of their band on the gel (Figure 5).

1. http://www.biotech.iastate.edu/publications/ed_resources/Laboratory_protocols.html
2. http://www.sumanasinc.com/webcontent/animations/content/pcr.html

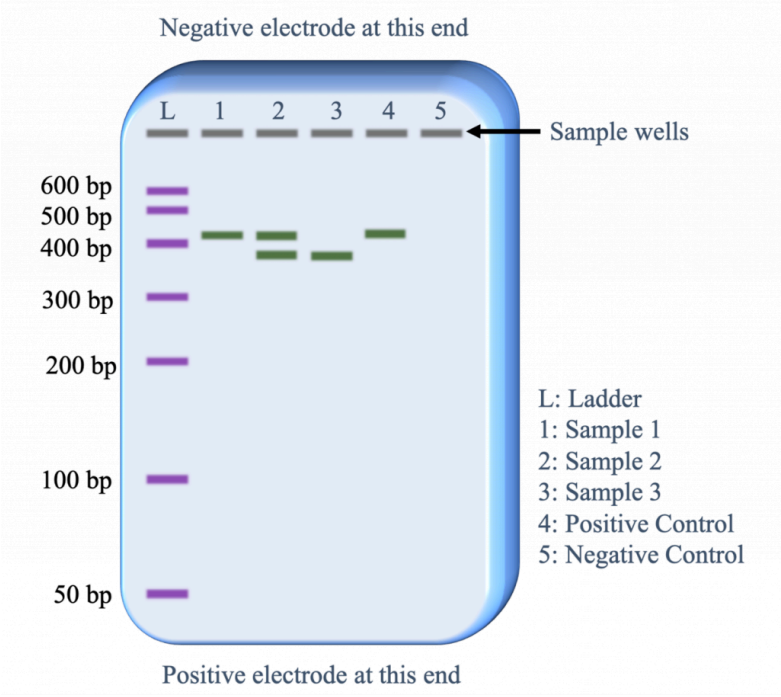


Figure 5. Visualization of PCR products on an agarose gel. A stain called ethidium bromide (EtBr) is added to the gel that binds to the DNA. When EtBr-treated DNA is exposed to UV light, it emits and orange-colored fluorescence. Image courtesy of Marjorie Hanneman, Iowa State University.

Example of the practical use of an SSR:

SSRs are used to track alleles that confer partial resistance to the soybean aphid. The *Rag1* allele that confers partial resistance to the soybean aphid is linked to an SSR made up of ATT repeats. In the soybean breeding program at Iowa State Univ., the parent line developed by the Univ. of Illinois that had the *Rag1* allele was crossed to an elite cultivar IA3027 [Crop Sci. 50:1891-1895 (2010)]. The SSR in the parent line that was linked to *Rag1* had 20 ATT repeats while the same SSR marker in IA3027 that was linked to the susceptible *rag1* allele had 26 ATT repeats (figure 6A). PCR primers were designed to amplify the SSR marker. When the amplified DNA was run on a gel, the band representing the shorter DNA fragment of 20 ATT repeats linked to Rag1 moved faster in the gel than the larger fragment with 26 ATT repeats associated with *rag1*. Samples (lanes) with one band in the same position as the *Rag 1* parent were considered homozygous for *Rag1*, those with one band in the same position as IA3027 were assumed to be homozygous for rag1, and those with two bands were considered to be heterozygous (Figure 6B).

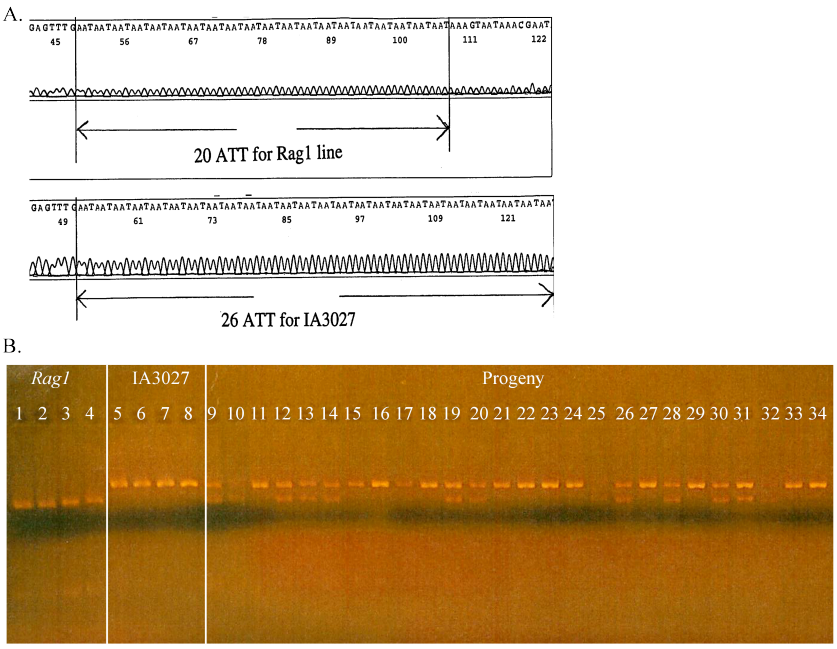


Figure 6. Molecular analysis of the Rag1 allele. (A) DNA sequences for the Rag1 parent line that has an SSR with 20 ATT repeats linked to the Rag1 allele and the cultivar IA3027 with 26 ATT repeats linked to the rag1 allele. (B) DNA bands for the Rag1 parent that was homozygous for the 20 ATT repeats linked to Rag1 (lanes1 to 4), for IA3027 that was homozygous for the 26 ATT repeats linked to rag1 (lanes 5 to 8) and their progeny (lanes 9 to 34). Progeny homozygous for the SSR with 20 ATT repeats (lanes 10, 25, 32), progeny homozygous for the SSR with 26 ATT repeats (lanes 15-17, 21-24, 27, 33 and 34), and the heterozygous individuals with both SSRs (lanes 9, 12-14, 19, 20, 26, 28, 30 and 31).

Example of the practical use of a SNP within a gene:

The DNA used as a SNP marker cannot be analyzed by gel electrophoresis in the same manner as an SSR because the base pair number and length of the DNA for two alleles will be the same. One method that is used to analyze SNPs is referred to as restriction fragment length polymorphism (RFLP).

There is a mutant allele in soybean *fan3*(A29) that controls a reduction in the linolenic acid content of the oil. Reduction of linolenic acid improves the shelf-life of the oil. The allele is one of those used in soybean cultivars for the production of oil with low linolenic acid. There is a single nucleotide difference between the *fan3*(A29) allele in A29 and the wild-type allele *Fan3* in the conventional cultivars such as Williams 82. The guanine (G) of the wild-type allele is changed to an adenine (A) in the mutant allele (Figure 7A). There is a an enzyme found in a bacterium, referred to as a restriction enzyme, that cuts the DNA of soybean containing the wild-type allele, but not the mutant allele, specifically at the location of the SNP. As a result, the DNA of a homozygous wild-type plant has two fragments (bands) when run on a gel, a homozygous mutant plant has one band, and a heterozygous plant has three bands (Figure 7B).

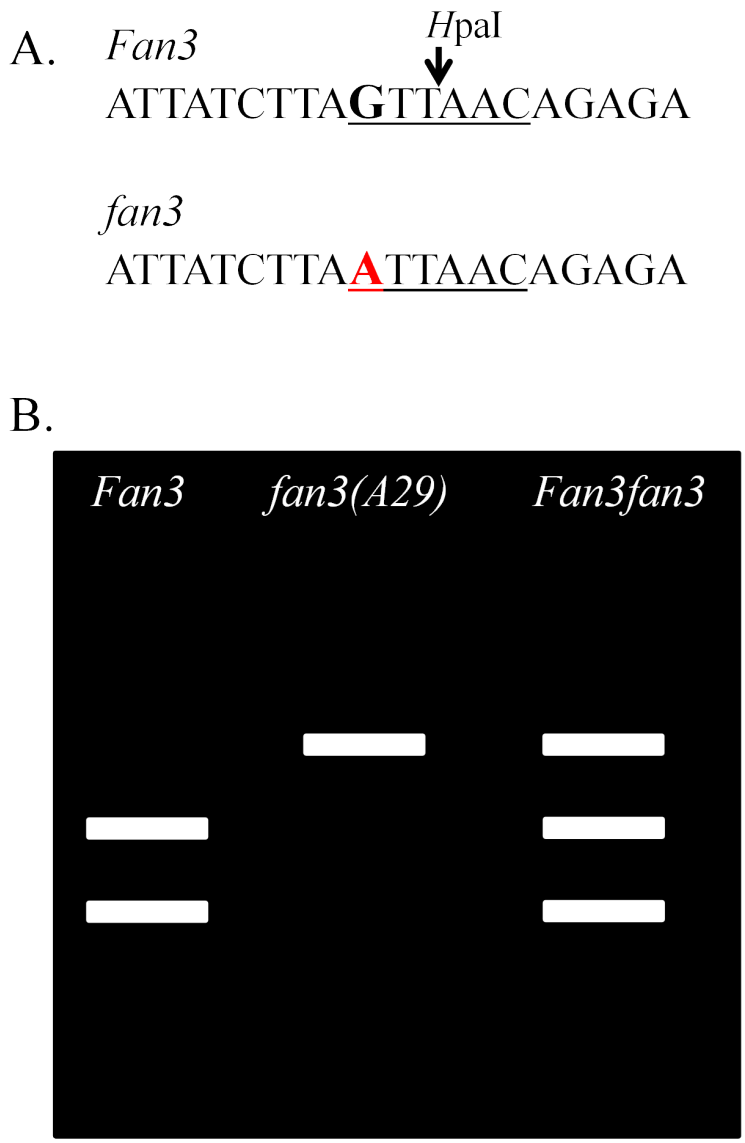


Figure 7. Analysis of a SNP for the Fan3 and fan3(A29) alleles in soybean. (A) The Fan3 allele has a G and the fan3(A29) allele has an A. The HpaI restriction enzyme recognizes a GTTAAC sequence in the Fan3 allele but not the ATTAAC in the fan3 allele. The HpaI restriction enzyme cuts the DNA of the Fan3 at the site marked by an arrow (B). An illustration of the DNA banding pattern after restriction enzyme digestion of DNA from an individual that is homozygous for the Fan3 allele (lane 1), an individual homozygous for the fan3(A29) allele (lane 2), and a heterozygous individual with both the wild-type and mutant alleles (lane 3).

There are other methods of evaluating SNPs, including the SimpleProbe and Taqman assays. Those methods and others will be covered in a later course.

Novel traits frombiotechnology

Genetic engineering has been used successfully to develop novel genes of economic importance that can be used by the plant breeder. The genes may be isolated from one organism and transferred to another or may be genes of one species that are modified and reinserted into the same species. The new genes, commonly referred to as transgenes, are inserted into a plant by a process called transformation. The DNA used for transformation is referred to as a construct that has four essential components linked together (figure 8).

1. *DNA of the gene of interest*.
2. *A promoter that acts to turn the gene on and off in the cell*. The CaMV 35s promoter from the cauliflower mosaic virus (CaMV) is commonly used in genetic engineering. Other types of promoters, such as, the nopaline synthase promoter (NOS-Pro) also may be used to express transgenes in plant tissues.
3. *A selectable marker that is used to select cells that successfully obtained the construct during the transformation process.* In figure 8, the selectable marker in the construct is NPT II (Kan^r) that controls resistance to the antibiotic kanamycin. The cells of the plant used for transformation will be grown on a media containing the antibiotic. Other selectable markers that have been used successfully in plants include genes controlling herbicide resistance. Additional information about selectable markers used in plant genetic engineering can be found on pages 196-198 of the Journal of Biotechnology 107: 193-232 (2004).
4. *A terminator sequence, such as the nopaline synthase (NOS) gene is included to mark the end of the transgene sequence for proper expression in plant cells*.

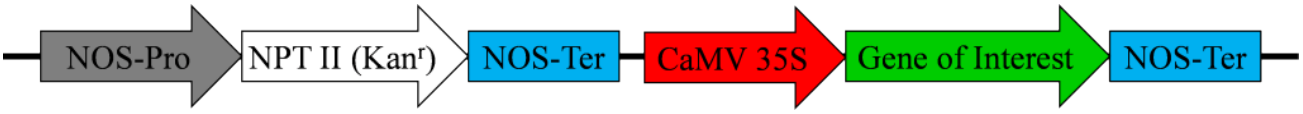


Figure 8. The basic elements of a transgene construct.

At present, very few host cells receive the construct during the transformation process. Each random insertion of the construct into the genome of plant cells is referred as an event. Useful events are rare because of the random nature of the transformation process. Selectable markers are very important because they allow the identification of the rare events (Figure 9). Scientists have to screen a large number of potential transformants to identify events that are useful for breeding.

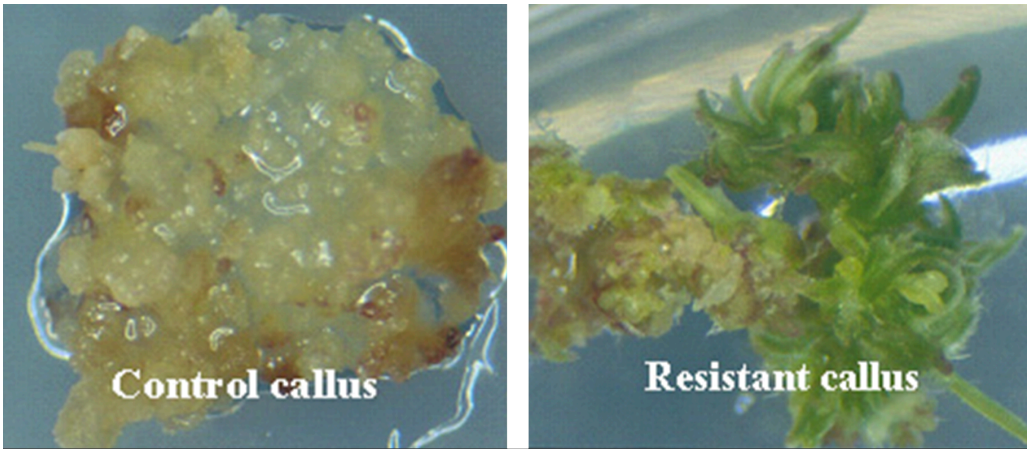


Figure 9. Selection for herbicide tolerance in buffalograss transformed with the gene that makes plants tolerant to glyphosate. The control calli lacking the glyphosate tolerance gene are killed by the herbicide that is part of the media on which the cells are grown (left panel). The calli in the right panel grew from a cell that received the glyphosate resistance gene during transformation and survived on the media. Photos courtesy of Shui-Zhang Fei, Iowa State University.

Plant transformation and tissue culture

The process of transformation involves the insertion of the desired transgene construct (figure 8) into cells of the recipient plant species. In this process, scientists isolate tissue or cells from the cultivar they wish to transform and use one of several methods to insert the transgene into the tissue or cells. Two commonly used transformation methods include *Agrobacterium tumefaciens*-mediated transformation and biolistics transformation, commonly referred to as particle bombardment. The biolistics method involves the use of high pressure to propel tungsten or gold beads coated with DNA of the gene construct into plant cells.

Agrobacterium is naturally found in soils and can infect many plant species by inserting its DNA into plant cells causing a disease called crown gall. In the early days of genetic engineering, scientists became interested in *Agrobacterium*'s ability to insert its DNA into plant cells. They subsequently modified the *Agrobacterium* by removing its ability to cause disease but retaining its ability to transfer foreign DNA into plant cells. The steps in *Agrobacterium*-mediated transformation of plants are described in figure 10.

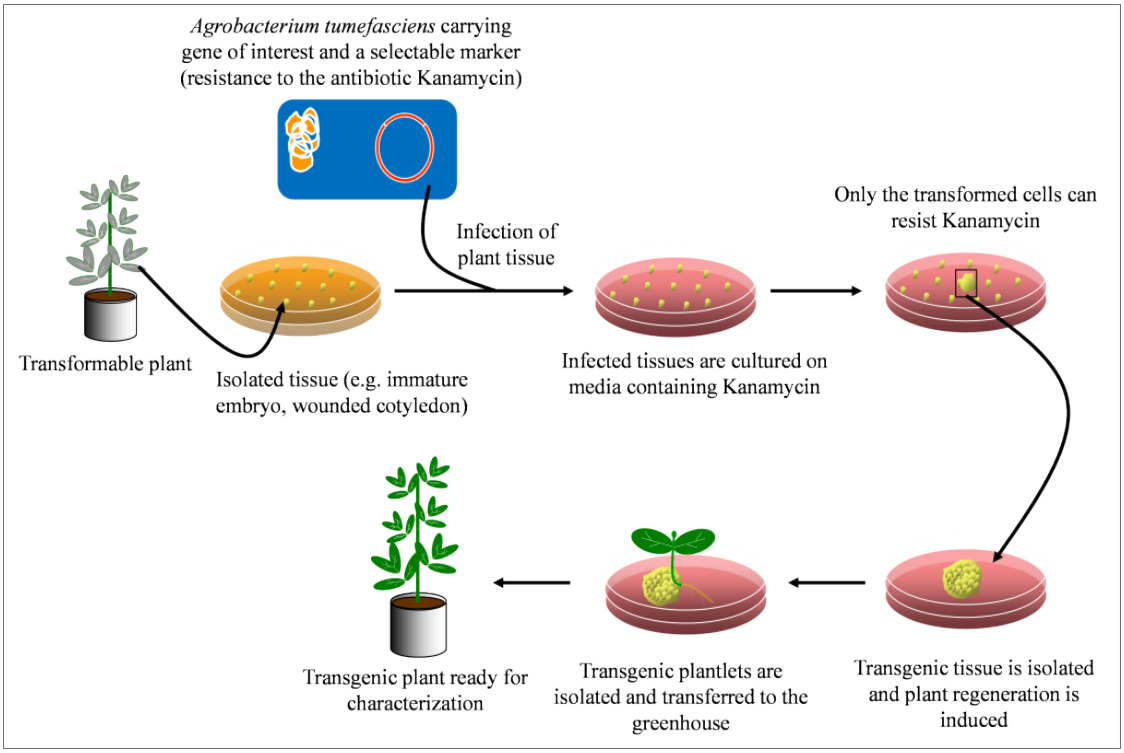


Figure 10. Transferring genes into plant cells by the Agrobacterium method. After infection of select tissue with the bacterium carrying a transgene construct with an antibiotic resistance gene as the selectable marker, the tissue is grown in a medium that contains the antibiotic that will kill all untransformed tissues or cells. Therefore, only tissues whose cells have been transformed with the transgene construct survive in the presence of the antibiotic. The surviving tissue is removed from the antibiotic and allowed to regenerate into whole plants. Normally, transgenic plants will be monitored in a controlled environment such as a growth chamber or greenhouse before they are grown in the field (Illustration provided by Kan Wang, Crop Bioengineering Center, Iowa State University).

The two most common examples of novel genes developed by genetic engineering that currently are used in commercial cultivars are insect resistance and herbicide tolerance.

Genes for insect resistance

Novel genes for insect resistance and herbicide tolerance have been obtained from other species and introduced into plants. For example, *Bacillus thuringiensis* (Bt) is a bacterium that produces crystal proteins that when ingested are toxic to the larvae of a number of insects. The gene that produces the crystal protein was isolated from Bt and was modified so that it could be better expressed in plant tissues. Transgenic plants expressing this bacterial toxin are commercially available. The steps involved in the engineering of Bt crops are illustrated in figure 11.

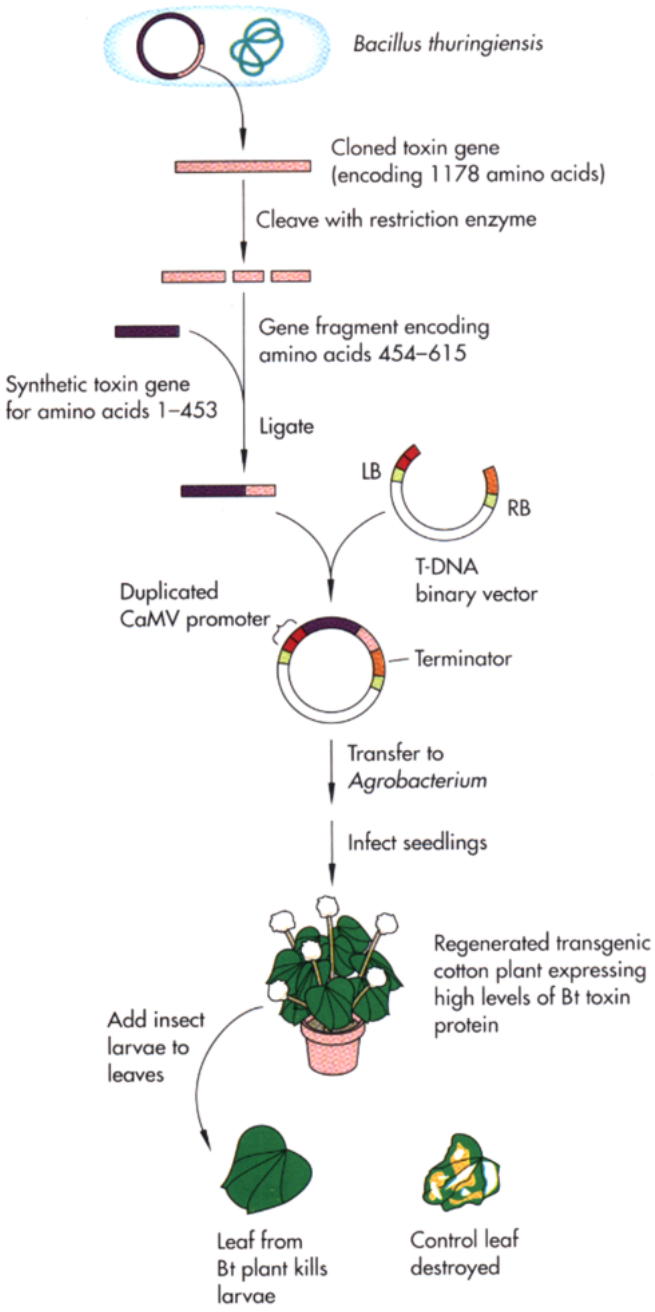


Figure 11. A scheme of genetic engineering and plant transformation steps to introduce insect resistance in cotton. The original bacterial toxin gene cloned from Bt was modified by combining it with a DNA fragment that was synthesized in the lab. The T-DNA binary vector allows the transgene to be introduced and multiplied into many copies inside the Agrobacterium and subsequently transferred into plant cells. A duplicated CaMV promoter is a modified version of the 35S CaMV promoter for stronger expression of the Bt toxin gene inside plant cells. Adapted from Watson, J.D., Gilman, M., Witkowski, J., & Zoller, M. (1997). Recombinant DNA. New York,

NY: Scientific American Books.

An example of transgenic insect-tolerant cultivars can be found at the following:

- Bayer Corn Rootworm Protection[3]

Genes for herbicide tolerance

When high levels of tolerance to a herbicide cannot be obtained by conventional breeding methods, other sources of tolerance such as bacterial genes have been be used to genetically engineer herbicide tolerance. For example, tolerance to glyphosate, the active ingredient in the herbicide Roundup, was obtained by inserting into plants a gene from a bacterium that was found to be resistant to the herbicide (figure 12). The 5-enol-pyruvylshikimate-3-phosphate synthase (EPSP) enzyme in plants is required for the production of certain amino acids. The EPSP enzyme is destroyed by glyphosate and the plant dies. The bacterial EPSP enzyme is not destroyed by glyphosate; therefore, plants that contain the bacterial EPSP are tolerant to the glyphosate.

3. https://www.cropscience.bayer.us/traits/corn-rootworm-protection/

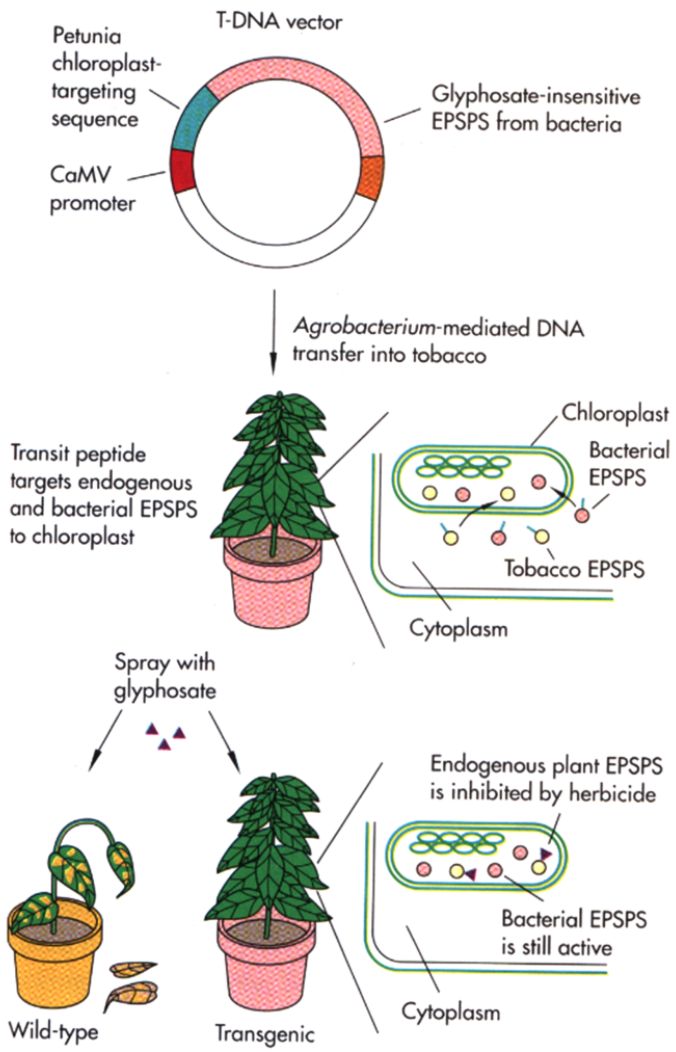


Figure 12. Steps in the genetic engineering of glyphosate tolerance in plants. A glyphosate-insensitive EPSPS from a bacterium is joined together with a DNA sequence that directs the delivery of the enzyme to the chloroplast. Agrobacterium transformation of plants with the bacterial gene results in plants with tolerance to glyphosate. Adapted from Watson, J.D., Gilman, M., Witkowski, J., & Zoller, M. (1997). Recombinant DNA. New York, NY: Scientific American Books.

Another important herbicide tolerance trait is resistance to glufosinate. Glufosinate is the active compound in the herbicide Liberty. Transformed glufosinate-tolerant crops contain either the *bar* or *pat* transgenes that can destroy glufosinate and decrease the ability of the herbicide to kill the plant. Both genes were isolated from bacteria.

An example of transgenic herbicide tolerant crops can be found at the following:

- Roundup Ready Corn 2[4]

Inheritance of a transgene in plants

Transformation is successful when a transgene is incorporated into one of the chromosomes. The cells that have only one copy of the transgene in their genomes are said to be **hemizygous** (hemi = half, zygous = zygote). Because the segregation in the progeny of a hemizygous plant is the same as for a heterozygous plant, the term heterozygous will be used in this course when referring to a plant that is not homozygous for the transgene. The trait will segregate in the progeny in the same manner as any other gene in the plant as illustrated below (Figure 13).

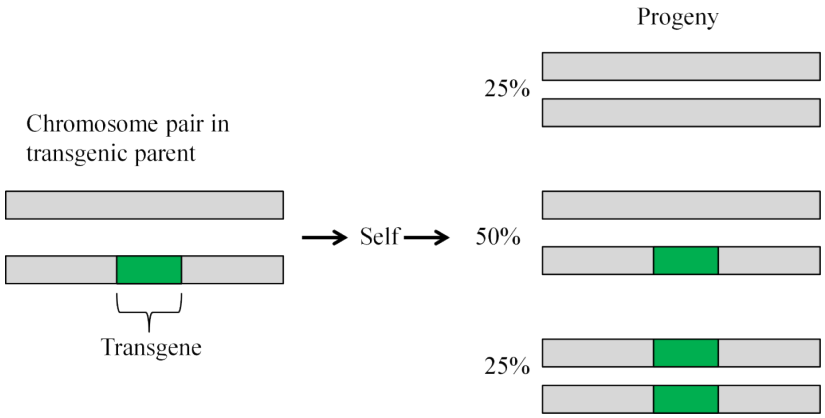


Figure 13. The ratio of possible offspring when a hemizygous diploid plant is self-pollinated.

The tissue culture process of regenerating transgenic plants from callus may result in genetic variation that is not associated with the transgene. Also, the parent line used for transformation commonly is selected for the frequency with which useful events can be obtained and not its agronomic performance. Therefore, transgenes are incorporated into commercial cultivars by conventional breeding procedures, such as backcrossing.

Review Questions

1. Describe a hypothetical or real trait, other than those discussed in the text above, for which it would it be

4. https://www.cropscience.bayer.us/traits/corn/roundup-ready-corn-2/

an advantage to use marker-assisted selection (MAS) in a breeding program, instead of phenotypic selection?

2. Describe a hypothetical or real trait, other than those discussed in the text above, when MAS would be less desirable to use than phenotypic selection?

3. Describe the hypothetical DNA sequence of two parents that are polymorphic for an SSR that could be used to track two alleles of a gene of interest. Draw a picture of how the two SSR alleles would look on a gel after electrophoresis. Be sure to label the negative and positive poles of the gel.

4. Describe the hypothetical DNA sequence of two parents that are polymorphic for a SNP that could be used to track two alleles of a gene of interest.
   a. What are two major advantages of a SNP in a functional gene compared with a linked SNP?

5. Describe the DNA sequence at the restriction site for a specific restriction enzyme that you could purchase from a supplier. Illustrate the difference in the DNA sequence of two alleles that can be detected with the use of restriction enzyme. Draw a picture of how the two alleles would look on a gel after electrophoresis. Be sure to label the negative and positive poles of the gel.

6. Below is a gel profile of an SSR marker linked to the *Rag1* allele for partial resistance to the soybean aphid resistance for four parents, P1 (lane 1), P2 (lane2), P3 (lane 3), and P4 (lane 4). P3 was used as the female parent in crosses to each of the other parents (P1, P2, and P4). Lanes 6 through 10 are the gel results for the plants that grew from seed produced by artificial hybridization, some of which may be the result of accidental self-pollination.

   Answer the following questions using the agarose gel picture and the sequence data below.

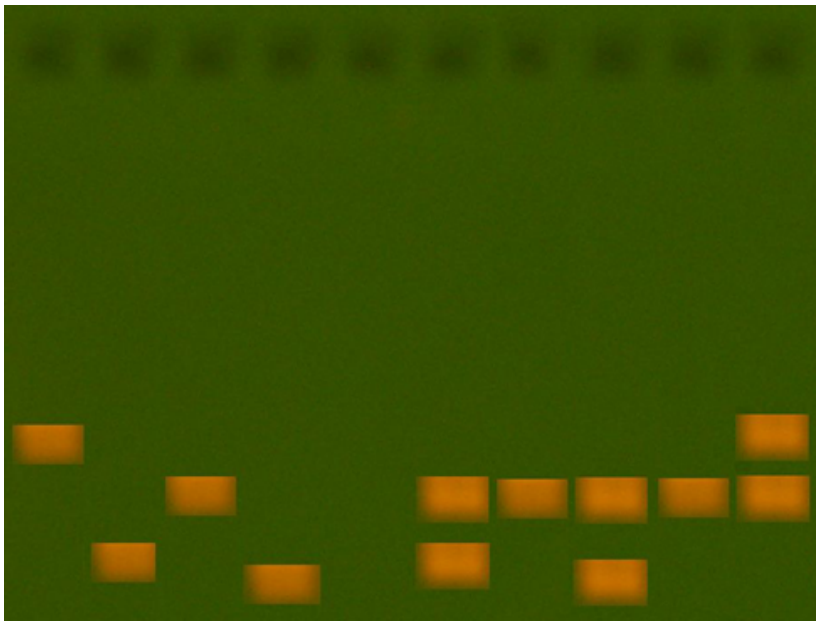
   a. What is the number of the three-base repeats for each parent based on the sequence data?

   b. Describe the relationship between the number of repeats in each parent and the distance its DNA traveled on the gel during electrophoresis.

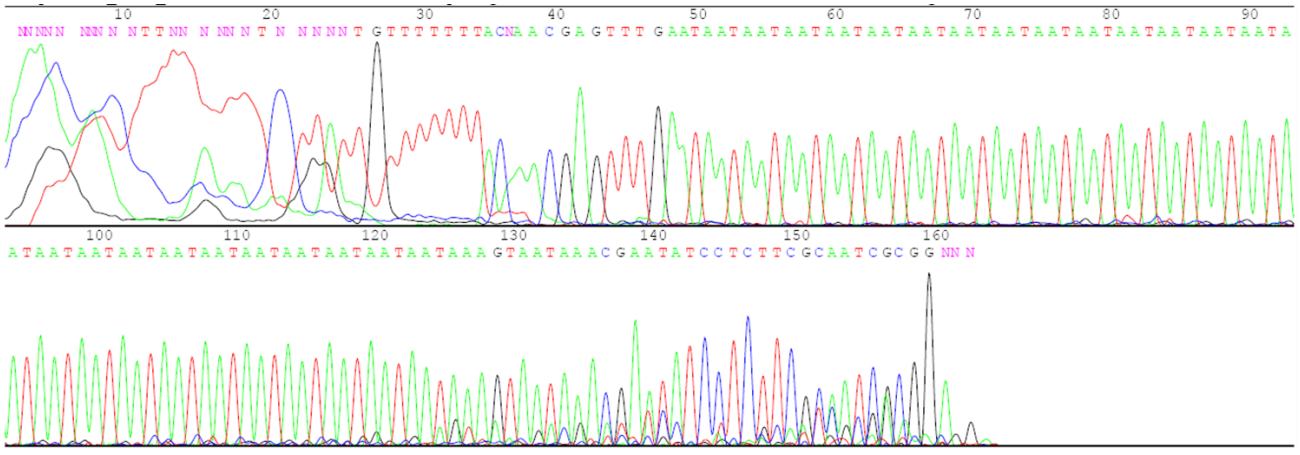
   c. Determine which plants in lanes 6 to 10 are F_1 hybrids and which are accidental selfs.

   d. Indicate which parents were crossed to create each of the F1 hybrids.

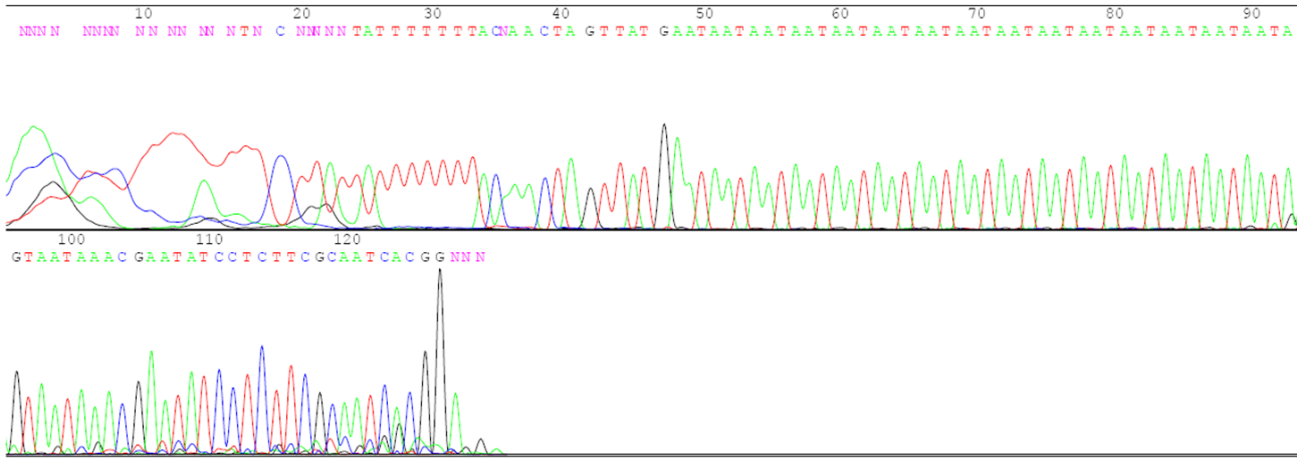
e. If the F_1 hybrid for lane 6 is selfed to produce F_2 progeny, how many different gel patterns will be observed in the F_2 progeny? Describe the gel patterns and their frequencies.



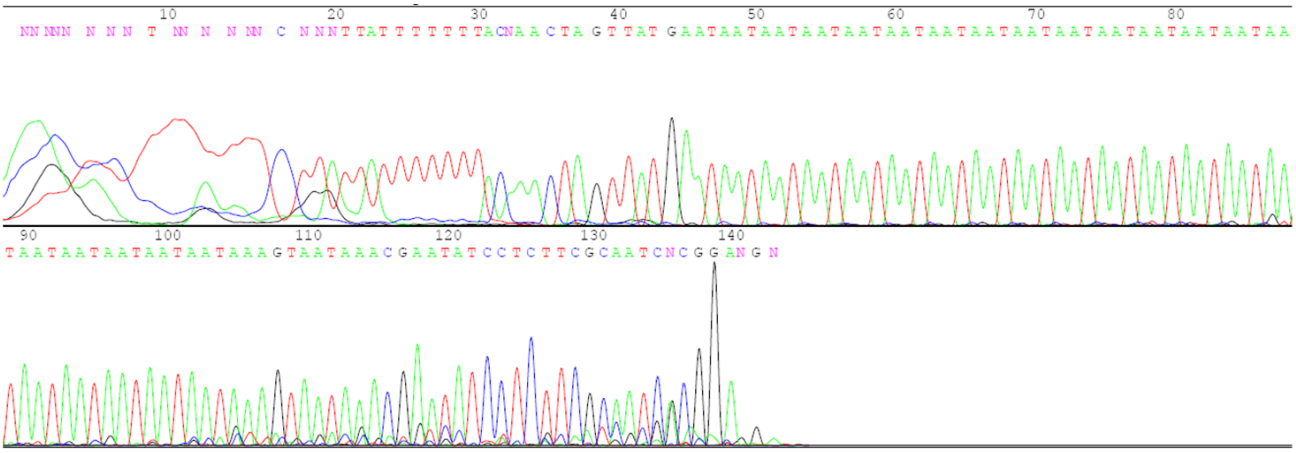
Agarose Gel: DNA migration moved from the top to bottom of the image. There are ten columns you can count across the base.



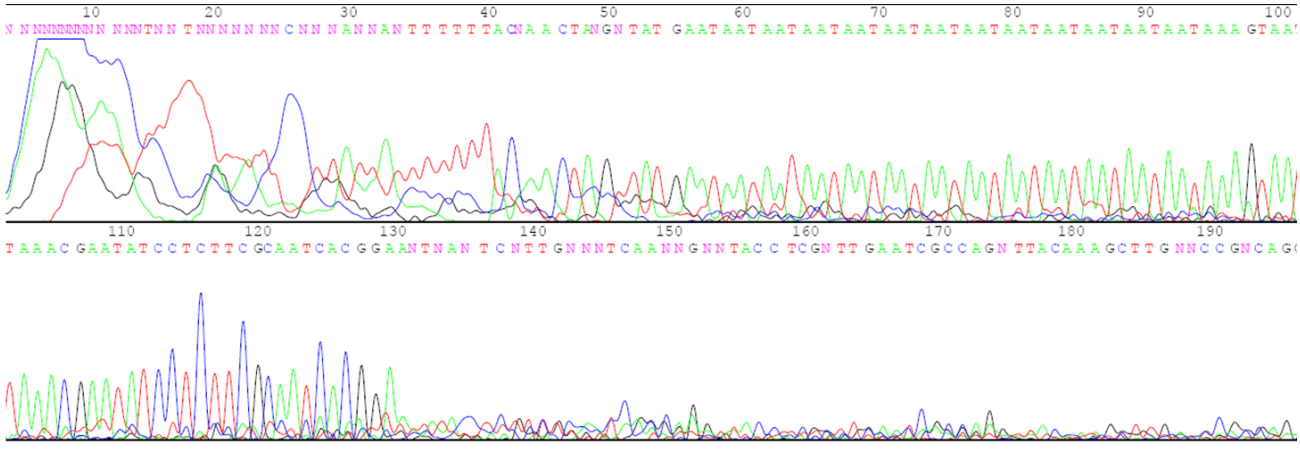
Lane 1



Lane 2



Lane 3



Lane 4

Backcrossing

Walter R. Fehr and Walter P. Suza

Readings:

- Chapter 28: Backcross Method [pdf], *Principles of Cultivar Development. Vol. 1: Theory and Technique*, by Walter R. Fehr (Access the full book)

Introduction

Backcrossing is used extensively to incorporate alleles for novel traits into elite germplasm for a cultivar development program. The novel alleles may be natural mutations or may be the result of mutagenesis or genetic engineering.

The strategy for conducting a backcrossing program is dependent on the type of cultivar being developed.

Pure-line and hybrid cultivars

Chapter 28 in *Principles of Cultivar Development* provides a thorough discussion of the principles involved in conducting a backcrossing program for plant species in which novel genes are incorporated into elite germplasm that will be used as pure-line cultivars or as inbred lines for hybrids. The goal of a backcrossing program is to recover a pure line or inbred that will contain the novel allele and be as good as the recurrent parent for all other important traits. Table 1 provides an illustration of a backcrossing program at Iowa State University to incorporate a gene for aphid resistance into the soybean cultivar IA3027.

It is common in commercial pure-line and hybrid cultivars to pyramid or stack multiple novel alleles. For pure-line cultivars, the novel alleles must be stacked together. For example, assume that the breeder wants to stack a novel dominant allele for herbicide resistance (H) with a novel dominant allele for insect resistance (I). The backcrossing program must lead to a cultivar with the genotype HHII.

For hybrid cultivars, it is possible to put one novel dominant allele, such as herbicide resistance (H), in one inbred line and a different dominant allele in another inbred line, such as insect resistance (I). When the two inbreds are mated to produce a commercial single-cross hybrid, the F1 plants grown by the farmer would be heterozygous HhIi and have both herbicide resistance and insect resistant.

Clonal cultivars

Heterozygosity is important for the performance of clonal cultivars. Self-pollination or the mating of related individuals results in homozygosity, **inbreeding depression**, and unacceptable performance. Because backcrossing to a single recurrent parent results in homozygosity, breeders of clonal cultivars generally change the elite parent every backcross generation. The procedure is referred to as modified backcrossing because there is not a single recurrent parent. The backcrossing program results in a population from which individuals with the novel allele and other desirable traits can be identified for subsequent clonal evaluation as potential cultivars.

Synthetic cultivars

Heterozygosity and heterogeneity are major considerations in a backcrossing program involving synthetic cultivars of forage and turf species. As with clonal cultivars, the breeder tries to avoid the inbreeding depression associated with homozygosity. This is accomplished by changing the elite parent each backcross generation. To adequately sample the genetic heterogeneity of synthetic cultivars, as many different plants as possible of the elite parent are used each backcross generation.

For autopolyploid species, such as alfalfa, it is not practical to incorporate a novel allele into every individual plant of a synthetic cultivar. Instead, the goal of the breeder is to have an adequate percentage of plants in a cultivar with the novel allele. For example, a transgene for glyphosate resistance, referred to as the Genuity® Roundup Ready® Gene, has been incorporated into synthetic cultivars of alfalfa. The majority of the plants in the synthetic cultivars will contain the transgene. The seeding rate for the cultivar can be increased by the farmer to account for the plants that will die when sprayed with the herbicide.

Managing a successful and efficient backcrossing program

Step 1. *Selection of appropriate donor and recurrent parents.*

The donor parent parent selected for the program should have as many traits that are similar to the recurrent parent as possible to minimize the number of backcross generations needed to recover a desirable cultivar. In the illustration provided in Table 1, LD05-15601 was considered by the breeder at the Univ. of Illinois to be the highest yielding line available in his breeding program that had the *Rag1* allele. Other traits that could have been considered in choosing the donor parent were seed size and protein content as similar as possible to that of IA3027.

The recurrent parent should be one that will continue to have competitive performance compared with other cultivars when the backcross is completed. The backcrossing program to develop IA3027RA1 began in 2006 and commercial quantities of seed were not available until 2010. IA3027 was used as the recurrent parent because the breeder believed in 2006 that the backcross-derived version with the *Rag1* gene would be a competitive cultivar in 2010. It is common for breeders to use as recurrent parents elite experimental lines that are likely to become cultivars in the future. This reduces the number of years between the commercial use of the original recurrent parents and that of the backcross-derived version. The disadvantage of this approach is that the backcross program may have to be discarded if the experimental line used as the recurrent parent fails to be accepted for commercial use.

Step 2. *Minimizing the number of backcross and self-pollinated seeds needed.*

The use of artificial hybridization to obtain backcross seed can be a limiting factor for some self-pollinated species. Chapter 28 has a table that is very useful for calculating the minimum number of plants or seeds needed to obtain the required number with the allele(s) of interest at the 95% or 99% probability level. Understanding how to make this calculation is critical for managing a successful program with the least amount of resources possible. The calculation requires that the breeder understand "q", the expected genotypic frequency of individuals with the desired allele(s); "r", the number of individuals with the desired allele(s) that the breeder needs; "p" the probability of successfully obtaining the "r" number of individuals; and the germination of the seeds when they are planted.

The number of backcross seed required is directly related to the number of alleles that are to be incorporated into the recurrent parent. When two or more alleles are involved, the breeder must decide whether to backcross both alleles individually and combine them together when backcrossing for each is completed or backcross them together in the same program. Less backcross seed is required each generation if individual programs are conducted for each allele; however, an additional season may be required to combine the alleles, if all of them are not in the original donor parent.

A breeder may choose to obtain more than the minimum number of seeds or plants indicated by the calculation in order to select among seeds or plants for genes of the recurrent parent. For example, if only 10 BC1F1 plants are needed for crossing, the breeder may choose to obtain 40 plants, genotype them with molecular markers, and choose the 10 for crossing that have the greatest frequency of markers that match the recurrent parent. This selection may reduce the number of backcross generations needed to recover the desired percentage of the recurrent parent genotype.

Step 3. *Minimizing the number of years to complete the backcross*

The likelihood that a backcrossed-derived version of the recurrent parent will be competitive is enhanced by utilizing as many seasons per year as possible for crossing. In some cases, this may require that backcrosses are made to plants before it is known if they have the desired allele(s), which means that backcross seed obtained from plants found to be without the desired allele would have to be discarded. Most breeders would prefer to do the extra work in order to reduce the number of years.

The use of multiple seasons a year for backcrossing can result in problems if environmental conditions in some seasons are not highly favorable for artificial hybridization. The program described in Table 1 encountered problems with obtaining hybrid seed in Puerto Rico and with adverse conditions during germination at Ames. It is not uncommon for a breeder to revise a backcross plan to deal with such unforeseen circumstances.

The number of years can be minimized by utilizing the fewest number of backcross generations as possible. This is strongly influenced by the similarity of donor and recurrent parent, the degree to which the backcross-derived version must perform like the recurrent parent, and the testing of the backcross progeny.

With regard to testing of backcross progeny, there are two alternatives.

- **Alternative 1:** The breeder may choose to grow multiple backcross-derived lines; select those that are similar for phenotypic traits with a high heritability; and bulk seed of the selected lines for release of the new cultivar. The advantage of this strategy is that it requires less time and resources than the second alternative. The disadvantage is that the new cultivar may not perform as well as the recurrent parent for

important quantitative traits. In the example of Table 1, a bulk of phenotypically similar lines was made without yield testing. It was found that the bulk did not perform as well as the recurrent parent.

- **Alternative 2:** For this alternative, replicated tests are conducted of the individual backcross lines, as was described in Table 1, and seed of only those lines with acceptable performance is bulked to form breeder seed of the new cultivar or inbred line.

The choice between the two alternatives is influenced by the experience of the breeder in working with the allele from the donor parent. If it is the first time the breeder has backcrossed with the allele, the decision may be to conduct replicated tests of individual backcross lines to determine their similarity to the recurrent parent for important quantitative traits. Also, replicated testing may be preferred when the donor and recurrent parent differ for multiple quantitative traits, as was the case for the example in Table 1. The donor parent LD05-156021was significantly lower than the recurrent parent IA3027 in seed size and protein content, both of which are quantitative traits. By testing the 30 individual lines, the breeder was able to discard 12 that had lower seed size and protein content than the recurrent parent.

Table 1. Development of the soybean variety IA3027RA1.

Year	Activity
2006	The cross of IA3027 x LD05-15621 was made in Puerto Rico during March to obtain F_1 seeds. The objective of the cross was to backcross the *Rag1* for aphid resistance from the line LD05-15621 into the cultivar IA3027. IA3027 was chosen as the recurrent parent because it had the highest yield of cultivars with large seed and high protein that are used by the soyfood industry. The donor parent LD05-15621 was developed by the Univ. of Illinois and IA3027 was developed by Iowa State University.
2006	The F_1 seeds were planted in the field at the Iowa State University Agricultural Engineering and Agronomy Research Center near Ames, IA, in May. The F_1 plants were crossed to IA3027 to obtain BC_1F_1 seeds.
2006	The BC_1F_1 seeds were planted during October in Puerto Rico for the next backcross; however, no BC_2F_1 seeds were obtained because of unfavorable environmental conditions. Leaves were harvested from each BC_1F_1 plant to identify plants heterozygous for the *Rag1* gene based on molecular analysis for a SSR linked to the gene. The heterozygous BC_1F_1 plants were harvested individually.
2007	The BC_1F_2 seeds from the heterozygous plants were planted during January in Puerto Rico. Each BC_1F_2 plant was evaluated with the SSR to identify plants homozygous for the *Rag1* gene. Five homozygous resistant plants were backcrossed to IA3027 to obtain BC_2F_1 seeds. The five homozygous resistant plants were harvested individually.
2007	The BC_2F_1 seeds and the five $BC_1F_{2:3}$ lines were planted in the field during May in Ames. Due to adverse weather conditions, none of the BC_2F_1 plants survived. The five $BC_1F_{2:3}$ lines were screened for aphid resistance in the greenhouse. Two lines with scores similar to the donor parent LD05-15621 were crossed to IA3027 to obtain BC_2F_1 seed.
2007	The BC_2F_1 seeds were planted during October in Puerto Rico. Each BC_2F_1 plant was evaluated with the SSR to confirm that they were heterozygous for the *Rag1* gene. The heterozygous plants were crossed to IA3027 to obtain BC_3F_1 seeds.
2008	The BC_3F_1 seeds were planted during January in Puerto Rico. Each plant was evaluated with the SSR to identify those that were heterozygous for the *Rag1* gene. The heterozygous plants were harvested individually.

Year	Activity
2008	The BC_3F_2 seeds were planted during May in Ames. Each plant was evaluated with the SSR to identify homozygous resistant individuals. In August, plants were scored for aphid resistance in the field when a natural infestation of the insect occurred. Plants with aphid resistance and maturity similar to IA3027 were harvested individually.
2008	The $BC_3F_{2:3}$ lines were planted individually in Puerto Rico for seed increase.
2009	The $BC_3F_{2:4}$ seed of each line was planted in the greenhouse during March to test for aphid resistance. A sample of seed from each of 30 $BC_3F_{2:4}$ lines was bulked for yield testing.
2009	**a**. The bulk of lines was evaluated for seed yield and other characteristics in the Iowa Specialty Test in three replications of four-row plots at five Iowa locations. The 30 $BC_3F_{2:4}$ lines also were tested individually in two replications at each of four locations. **b**. The $BC_3F_{2:4}$ lines were grown at Ames in a seed increase and each of them was harvested individually. Seeds of 18 lines with similar agronomic and seed characteristics as IA3027 in the yield trials were bulked as breeder seed of IA3027RA1.
2009/10	The variety was licensed to interested companies by the Iowa State University Research Foundation. The companies produced foundation seed in Argentina for planting in the Midwest during 2010.

Review Questions 1

You are going to backcross the *B* allele for increased beta-glucan content in oat seed from a donor parent into an elite pure-line cultivar with the genotype *bb*. You can differentiate *BB* and *Bb* seeds from those that are *bb* by analyzing the beta-glucan content of the part of the seed without the embryonic axis and saving the part with the embryonic axis for planting. You cannot differentiate *BB* and *Bb* seeds from each other with the test. For any seed you select, the part with the embryonic axis can be used to obtain a plant for crossing or self-pollination.

a. What characteristics would you consider important in selecting your donor and recurrent parent?

b. Which parent would you use as female to produce F_1 seed? Why?

c. You cross the F_1 plants to the recurrent parent. Would you prefer to use the F_1 plant as the male or female in the backcross cross to assure that a plant grown the following season that has the *B* allele is a BC_1F_1 and not a F_2 resulting from an accidental self-pollination? Would your answer be the same for every subsequent backcross generation when deciding whether to use the recurrent parent as the male or female for the backcross?

d. If you want to be 95% sure of obtaining 10 BC_1F_1 plants with the genotype needed to continue the backcrossing program and your germination percentage is 70%, how many BC_1F_1 seeds would you have to obtain?

e. You test the B_1F_1 seeds for beta-glucan content. What genotypes would you expect to find in the BC1F1 seeds and in what frequency? What would be the genotype of the seeds you select for planting?

f. You cross the selected BC_1F_1 plants to the recurrent parent. If you want to be 99% sure of obtaining 6 BC_2F_1 plants with the genotype you need to continue the backcrossing program and your germination percentage is 60%, how many BC_2F_1 seeds would you have to obtain?

g. You test the BC_2F_1 seeds for beta-glucan content. What genotypes would you expect to find in the BC_2F_1 seeds and in what frequency? What would be the genotype of the seeds you select for planting?

h. You self-pollinate the selected BC_2F_1 plants. What genotypes would you expect to find in the BC_2F_2 seeds and what would be their frequencies? If you want to be 95% sure of having at least 15 BC_2F_2 seeds with the genotype *BB*, how many BC_2F_2 seeds would you have to test?

i. How many BC_2F_2 plants would you have to grow to be 99% sure of finding at least 10 that have the genotype *BB*? What would be the average percentage of the recurrent parent in the BC_2F_2 plants?

j. How many progeny would you have to test from each BC_2F_2 plant to be 95% sure of identifying those that have the *BB* genotype?

Review Questions 2

You are responsible for developing pure-lines cultivars of barley for Minnesota. You have a high-yielding cultivar Ada to which you would like to add a major recessive allele for insect resistance (r) and a major dominant allele for fungal resistance (F). The two alleles are at independent loci. The r and F alleles are in different donor lines. There is a direct SNP marker that can be used to differentiate the r and R alleles. There are no molecular markers yet developed for the f and F alleles.

Outline your breeding program season-by-season for developing a backcross version of Ada with the genotype rrFF in the ***shortest possible time and with the least amount of work***. You have two seasons a year. Season 1 is in the field in Minnesota and season 2 is in the field in Arizona. At both locations, you can do hybridization and selfing in the field. All tests for insect or fungal resistance are done in the greenhouse in Minnesota. You

cannot produce enough hybrid seed on a plant to use that hybrid seed for progeny testing. However, you can have progeny test results before flowering occurs. Your laboratory results from molecular marker analysis are available before flowering.

In outlining your program, indicate for each season the number of plants grown, genotype of plants used for crossing, and number of hybrid seeds obtained on each plant. If progeny testing is involved, indicate the number and genotype of plants to be progeny tested and the genotype and number of their progeny that need to be evaluated. Use the following assumptions.

1. Begin the backcrossing program by making the single cross in Arizona

2. Probability of recovering the desired genotype is 0.95

3. Germination is 90%

4. Maximum number of hybrid seed that can be obtained on a plant is 12

5. Maximum number of selfed seed on a plant is 100

6. The new cultivar should have at least 90% of its genetic background from Ada.

7. You will not yield test before producing breeder seed

8. Breeders seed will be produced from a composite of 40 progeny rows that are homogeneous rrFF.

Inbreeding

Walter R. Fehr and Walter P. Suza

Readings:

- Chapter 8: Inbreeding [pdf], *Principles of Cultivar Development. Vol. 1: Theory and Technique*, by Walter R. Fehr (Access the full book)
- Chapters 22-27 [pdf], *Principles of Cultivar Development. Vol. 1: Theory and Technique*, by Walter R. Fehr

Introduction

Inbreeding for the development of homozygous and homogeneous lines is only important for the development of pure-line and hybrid cultivars. It is not used for the development of clonal or synthetic cultivars because self-pollination results in severe inbreeding depression.

It is possible to carry out inbreeding by half-sib or full-sib mating, instead of self-pollination. However, the number of generations required to reach homozygosity is more than through the use of self-pollination. As a result, half-sib or full-sib matings rarely are used to develop inbred lines for cultivar development. The chart in figure 1 illustrates the number of generations it may take to attain complete homozygosity by different mating systems.

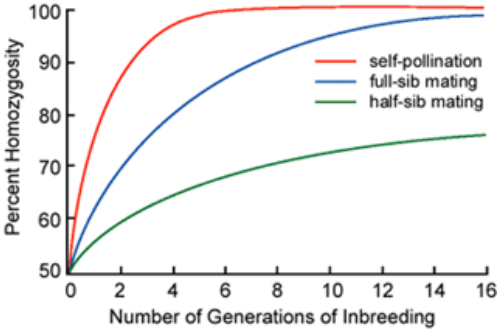


Figure 1. Number of generations required to reach homozygosity by self-pollination, full-sib mating and half-sib mating.

Factors influencing the choice of methods for obtaining pure lines/ inbred lines

Environments available

One of the key factors that influences the method of advancing a population from the F_1 to later generations is the environments available to the breeder. The environment for which a breeder is developing new cultivars will be referred to as the local environment. In most breeding programs today, a breeder will use off-season nurseries for generation advance, which include greenhouses, growth chambers, and locations where the environment is not the same as the local environment. For example, the wheat cultivar Select was developed by breeders in South Dakota for their local environment [JPR 5:196-201 (2011)]. For generation advancement

of the population from which the cultivar was developed, the breeders used Yuma, Arizona, for the off-season nursery. The possibility of effectively selecting among plants or lines in Yuma for traits of interest would influence how a population would be planted and harvested in Yuma.

The effectiveness of selection in an off-season nursery is highly dependent on the trait. Although seed yield commonly cannot be evaluated in off-season nurseries, selection for other traits may be possible. For example, the cranberry bean cultivar Bellagio was developed for Michigan [JPR 4:171-174 (2010)]. In the off-season nursery at Isabela, Puerto Rico, the breeders were able to select for upright vine, lodging resistance, good pod load, cranberry bean seed size and color, and freedom from diseases.

The two methods that are most influenced by the environment are the bulk and pedigree. In the **bulk method**, the seed planted in one generation is obtained by sampling from a bulk of seed harvested the previous generation. The likelihood that a plant will have one of it progeny represented in the sample used for planting is directly associated with the number of seeds the plant produced. An F_2 plant that produced 40 seeds would be less likely to be represented in the next generation than an F_2 plant that produced 200 seeds. If the number of seeds produced by plants in off-season nurseries was not representative of their production in the local environment, they could be eliminated from the population by use of the bulk method, even though they may have the potential to be a superior new cultivar or inbred. Use of the **pedigree method** is also highly dependent on the environment of the off-season nursery. If a trait is not adequately expressed in the off-season nursery, selection would be ineffective.

It is common for breeders to use a combination of methods during generation advance. For example, a breeder may select among F_2 plants in the local environment, harvest individual F_3 plants in an off-season nursery without selection, and resume selection among $F_{3:4}$ lines the following season in the local environment.

Cost

Half of the genetic variation among individuals in a population will be expressed in the F_2 generation. To maximize genetic variation for seed yield and other traits for which selection is not feasible during generation advance, it is ideal to have as many different F_2 plants and their progenies represented in the population when lines are selected for evaluation. The methods that can assure that every line derived from a population traces to a different F_2 individual are the pedigree, the single-seed procedure of **single-seed descent**, and the single-hill procedure of single-seed descent. Doubled haploid lines normally trace to individual gametes from an F1 plant, each of which are genetically different. These methods require more labor than the bulk or the multiple-seed procedure of single seed descent. The breeder must decide how to balance cost versus genetic variability in the lines derived from a population.

Generation in which lines are derived

Regardless of the method of generation advance used for a population, the breeder must decide at which generation lines will be derived for extensive testing as a potential cultivar or as an inbred in a hybrid. In selecting parents for mating, genetic differences in their parentage or in their molecular makeup are emphasized; therefore, there would be many heterozygous loci in the F_1 hybrid. As a result, complete homozygosity is rarely achieved in plants from which pure-line cultivars or inbreds are derived, with the exception of the doubled haploid method. Instead, the breeder derives lines at a generation when there is an adequate frequency of plants whose progeny will be sufficiently uniform in appearance and homogeneous for major genes of interest.

With multiple segregating loci, the proportion of homozygotes in various selfing generations can be estimated using the following formula: $[(2^m - 1)2^m]^n$: where *m* is the number of selfing generations (F_2 = 1; F_3 = 2) and *n* is the number of segregating loci. For example, an F_1 plant with four independent segregating loci will result in the following frequency of homozygous plants in F_2, $[(2^1 - 1)/2^1]^4$ 1/16 = 6.25%. The expected proportion of completely homozygous plants in F_2 and later generations of selfing for different numbers of segregating loci are indicated in table 1. The proportion of homozygotes decreases sharply with increasing heterozygosity in the F_1.

Table 1. Frequency of completely homozygous individuals in various selfing generations in relation to the number of segregating loci in F_1.

Number of segregating loci in the F_1	F_2	F_4	F_6	F_8
1	50.00	87.5	96.87	99.22
2	25.00	76.56	93.85	98.44
3	12.50	66.99	90.91	97.67
4	6.25	58.62	88.07	96.91
5	3.13	51.29	85.32	96.15
10	0.01	26.31	72.79	92.45
100	7.89×10^{-31}	6.1×10^{-4}	4.18	45.64
1000	9.33×10^{-302}	1.02×10^{-58}	1.63×10^{-14}	0.04

The generation in which a cultivar or inbred line is derived for commercial use is influenced by the degree of homozygosity and homogeneity required and the cost and time involved in each generation of selfing. The number of heterozygous loci is an F_1 hybrid generally is very large, which means that only a very small percentage of plants are completely homozygous when lines are derived (Table 1). As a result, pure-line cultivars and inbreds of hybrids rarely are completely homozygous and homogeneous, unless they were derived from doubled haploids. The breeder attempts to achieve the level of homogeneity that is required for commercial acceptance of the cultivar or inbred line. In general, breeders of pure-line cultivars commonly derive cultivars in an earlier generation than breeders responsible for developing inbreds of hybrids. For example, the hard red winter wheat cultivar Camelot originated from an F_3-derived line [JPR 3:256-263 (2009)]. In contrast, the maize inbred line GT603 was developed through seven generations of self-pollination [JPR 5:211-214 (2011)]. One reason for seeking more homozygosity and homogeneity in an inbred is to be able to recognize and remove off-type plants in a hybrid seed production field before crossing occurs. A second reason is that if two inbreds are crossed that are not adequately homozygous and homogeneous, undesirable segregation may occur in a commercial hybrid field.

Examples of how the inbreeding methods described in the text have been applied in development of cultivars and germplasm can be found in the *Journal of Plant Registrations*. In the following examples, the underlined sentences are comments that did not appear in the text.

Example 1: *Bulk method combined with mass selection*

Information found in JPR 5:151-155 (2011) describes the development of 'Gadsby' barley by use of the bulk

method and mass selection. The breeders developed Gadsby as a general-purpose barley with resistance to scald.

- Step 1. In 1997, the F_2 plants from a cross between the parents H92066001 and TR248 were grown in the field at Lacombe, Alberta, Canada. The plants were inoculated with scald by spreading straw that had been collected from a field infested with the disease the previous year. The F_3 seed of the population was harvested in bulk.
- Step 2. For mass selection, the F_3 seed was screened over a gravity table and the heavier seed was saved for planting. It was assumed by the breeders that the heavier seed was from F_2 plants with resistance to scald and the lighter seed was from susceptible genotypes.
- Step 3. In 1998, the selected F_3 seed was planted in the field and the first two steps were repeated again. The same two steps were repeated with the F_4 bulk in 1999 and the F5 bulk in 2000.
- Step 4. In 2001, the F_6 bulk was grown in 2001 at two locations and a head from each of multiple individual plants was harvested and threshed separately. The progeny of each plant was evaluated in subsequent generations as F_6-derived lines; therefore, Gadsby was an F_6-derived cultivar.

The purpose of mass selection is to increase the frequency of individuals with a desired trait in a population. By infecting the plants each generation and selecting the heavier ones for planting, the breeders of Gadsby assumed that the frequency of F_6 plants with resistance to scald would be greater than if the bulk method had been used without infection or mass selection.

Mass selection also can be used with single-seed descent. For example, if a disease caused resistant plants to look different than susceptible ones, one or a few seeds could be harvested from the resistant ones to plant the next generation.

Mass selection is not expected to be perfect in selecting desirable seeds or plants. In the breeding of Gadsby, some of the susceptible plants may have escaped infection and produced seed as heavy as the resistant ones. As a result, some of the seed planted the next generation may have been from susceptible plants. Even though it is not perfect, it can potentially increase the frequency of desirable individuals in a population.

It is important to recognize that the term “mass selection” when used in connection with inbreeding a population has a very different meaning than when used in connection with recurrent selection (Lessons 13 and 14 of this course). When a population is self-pollinated, as in the case of Gadsby barley, the frequency of homozygous individuals increases each generation of mass selection. When mass selection is carried out for recurrent selection, selected plants are intercrossed, which maintains the heterozygosity of individuals.

Example 2: *Single-seed procedure of the single-seed descent*

The hard red spring wheat cultivar Duclair was developed by use of the single-seed procedure of single-seed descent [JPR 5:349-352 (2011)]. Some of the information provided below is not in the article and was obtained directly from the senior author.

- Step 1. In the greenhouse, the breeders crossed the cultivar Choteau with the line MT0249 to form a single-cross population.

- Step 2. The F_1 plants were grown in the field and F_2 seed was harvested.
- Step 3. The F_2 seed were planted in the greenhouse in September. One head from each plant was removed. The plants in the greenhouse are small and tillering generally does not occur; therefore, the plants can be distinguished from each other. One head that usually contains four to eight seeds is removed from each plant. One healthy seed from each head is bulked together for planting the next generation. The heads are stored as a reserve in case something destroys the subsequent planting.
    - Step 3. The F_3 seeds were planted in the greenhouse. Each plant was harvested individually to form an $F_{3:4}$ line.
- Step 4. The $F_{3:4}$ lines were planted in Bozeman, MT, during May. Individual lines were selected and single plants were harvested from the selected rows. The authors refer to the $F_{3:4}$ headrows as F_4 head rows. In their system of nomenclature, the row is described by the generation of the plants that are growing and does not describe when the line was derived.
    - Step 4. The $F_{4:5}$ lines (F_5 headrows) were planted for evaluation. The F_4-derived lines were evaluated in subsequent generations. One of the F_4-derived lines became the cultivar Duclair.

Example 3: *Multiple-seed procedure of single-seed descent*

The breeders of 'UA 4910' soybean referred to their method of generation advance as modified single-pod descent [JPR 5:49-53 (2011)]. In the text for this course, their procedure is called the multiple-seed procedure of single-seed descent. When the term "modified" is used for a method of generation advance, it means that the breeders modified what they considered the standard way that a method would be carried out. The only way to understand what was modified is to carefully ready what they did in comparison to what would be the standard procedure.

- Step 1. In 1997, the cross of Asgrow A4715 to DP 3478 was made in the field at Fayettevile, AR, to obtain a single-cross population.
- Step 2. The F_1 plants were grown in the 1997-1998 winter nursery in Costa Rica and F_2 seed was harvested.
- Step 3. In 1998, the F_2 population was grown in the field and two to three pods each containing two or three seeds were harvested from about 600 plants and the pods were threshed in bulk. Part of the seed was planted in Costa Rica (season 2).
- Step 4. The F_3 plants were grown in the 1998-199 winter nursery in Costa Rica. Two to three pods each containing two or three seeds were harvested from the plants and threshed in bulk.
- Step 5. The F_4 seed was planted in Keiser, AR, during 1999, and 60 plants from the population were harvested and threshed individually. The progeny of the plants were evaluated as F_4-derived lines in subsequent generations; therefore, UA 4910 was an F_4-derived cultivar.

Example 4: *Pedigree method*

Georgia-09B peanut cultivar was developed by use of the pedigree method with a backcross population [JPR 4:175-178 (2010)].

- Step 1: The cultivar Georgia Green was crossed to the line GA 942004. The F_1 generation was grown to obtain F_2 seed. F_2 plants were grown and harvested individually. A sample of seed from each F_2 plant was evaluated for oleic acid concentration in the oil and one of them was chosen for crossing as a $F_{2:3}$ line.
- Step 2: In 1998, the $F_{2:3}$ line was backcrossed to the recurrent parent Georgia Green to obtain BC_1F_1 seed. The goal of the backcross was to develop a population that had individuals with the desirable agronomic traits of Georgia Green and the high oleic and low linolenic acid traits of the donor parent GA 942004.
- Step 3: The BC_1F_1 seed was planted at the Univ. of Georgia Coastal Plain Experiment Station and the BC_1F_2 seed was harvested.
- Step 4: The F_2 seed was space planted and individual plants were selected based on plant and seed characteristics. The BC_1 designation used by the authors to precede the F designation of every generation will be left off in the following description so that the generation designations will be the same as used for other population types, such as a single cross or three-way cross.
- Step 5: The selected F_2 plants were grown as $F_{2:3}$ lines in two-row plots. Lines with desirable traits were selected and one or more individual F_3 plants with desirable traits were selected from within the selected lines.
- Step 6: The selected F_3 plants were grown as $F_{3:4}$ lines in two-row plots and one or more desirable F_4 plants were harvested individually from selected lines. The $F_{3:4}$ lines that traced to single plant selections from the same $F_{2:3}$ row the previous season would be grown in adjacent rows and referred to as a family. In the standard pedigree method, the breeder would first select the best families. Second, the best $F_{3:4}$ line(s) within the selected families would be identified. Third, the best individual F_4 plants within the selected lines would be harvested and threshed individually.
- Step 7: The selected F_4 plants were grown as $F_{4:5}$ lines in two-row plots. The best lines were selected and threshed in bulk to obtain $F_{4:6}$ seed. As in the step 6, the breeder would select the best families, followed by selection of the best line(s) within the selected families.
- Step 8: The F_4-derived lines were evaluated in replicated tests for three years. Georgia 09B was a F_4-derived cultivar.

Example 5: *Early-generation testing of selfed lines*

The developers of 'Barlow' hard red spring wheat described their inbreeding procedure as a combination of the modified bulk and pedigree methods. The process also involved early generation testing of selfed lines [JPR 5:62-67 (2011)]. This example is a good illustration of how breeders use combinations of methods to advance a population.

- Step 1: The cross of the line ND 744 to the line ND 721 was made in the fall greenhouse at North Dakota State University in Fargo, ND.
- Step 2: The F_1 seeds of the single-cross population were grown in the greenhouse in the spring of 2000.
- Step 3: The F_2 population was planted in the field during the summer of 2000. Spikes were harvested

from 200 plants selected for their reaction to leaf rust, plant vigor, height, and earliness. Twenty of the spikes were selected and threshed individually. Selection among individual F_2 plants is the first step in the pedigree method.

- Step 4: The 20 F_3 headrows ($F_{2:3}$ lines) were grown in Christchurch, New Zealand, during the winter of 2000-2001. Desirable lines were selected and each line was threshed in bulk to obtain $F_{2:4}$ seed. In the typical pedigree method, one or more individual F_3 plants would have been harvested from the selected lines. The breeders used the term "modified bulk" because they harvested each $F_{2:3}$ line in bulk, instead of harvesting individual plants.
- Step 5: The selected $F_{2:4}$ lines were planted in a yield test during the summer of 2001. A spike from 10 individual F_4 plants were harvested from the $F_{2:4}$ lines with desirable yield and other agronomic traits. The evaluation of the $F_{2:4}$ lines in replicated tests for quantitatively inherited traits represented an early generation test. It is considered an early generation test because the $F_{2:4}$ lines were not adequately homozygous and homogeneous to merit release as cultivars per se. The purpose of the test was to select lines for quantitative traits based on replicated tests whose progeny had the potential to become cultivars.
- Step 6: The seed from each of the ten spikes was planted in a headrow ($F_{4:5}$ line) in New Zealand during the winter of 2001-2002. One $F_{4:5}$ line was selected and threshed in bulk. This F_4-derived line was tested in subsequent generations and became the cultivar Barlow. In the standard pedigree method, the 10 F4:5 lines that were derived from the 10 individual spikes of each selected F2:4 line the previous season would be considered a family. The breeder would select the best families, followed by selection of the best line(s) within the best families.

Example 6: *Development of lines by the soybean breeding program of Iowa State University.*

The following pictures taken by Walter Fehr illustrate one method used by the soybean breeding program at Iowa State University to develop lines for evaluation as potential cultivars.

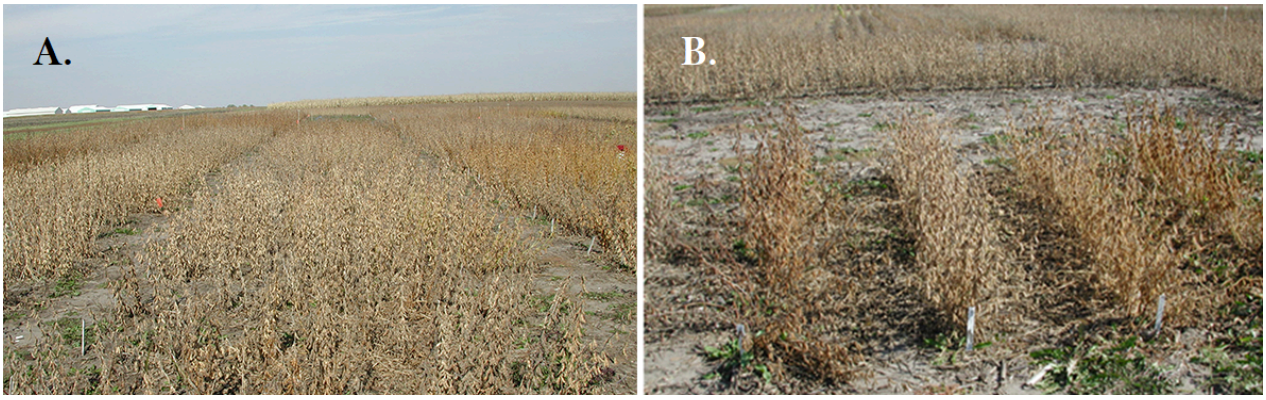


Figure 2. **A.** Crossing nursery of the soybean breeding program at Iowa State University. The rows of the parent lines 11 feet long are divided into three planting dates to spread out the flowering period. **B.** Individual rows of parent lines to be used for crossing in the soybean breeding program at Iowa State University. Both paired and unpaired parents are used as appropriate.

In this example, the crosses are made at Ames during the summer, the F_1 seeds are planted near Santa Isabel, Puerto Rico, the middle of October, the F_1 plants are harvested in January, the F_2 seeds are planted in early February, the F_3 seeds are harvested in May by the multiple-seed procedure of single-seed descent, the F_3 seeds are planted at Ames in May, and individual F_3 plants are harvested individually in the fall to form F_3-derived lines for subsequent evaluation.

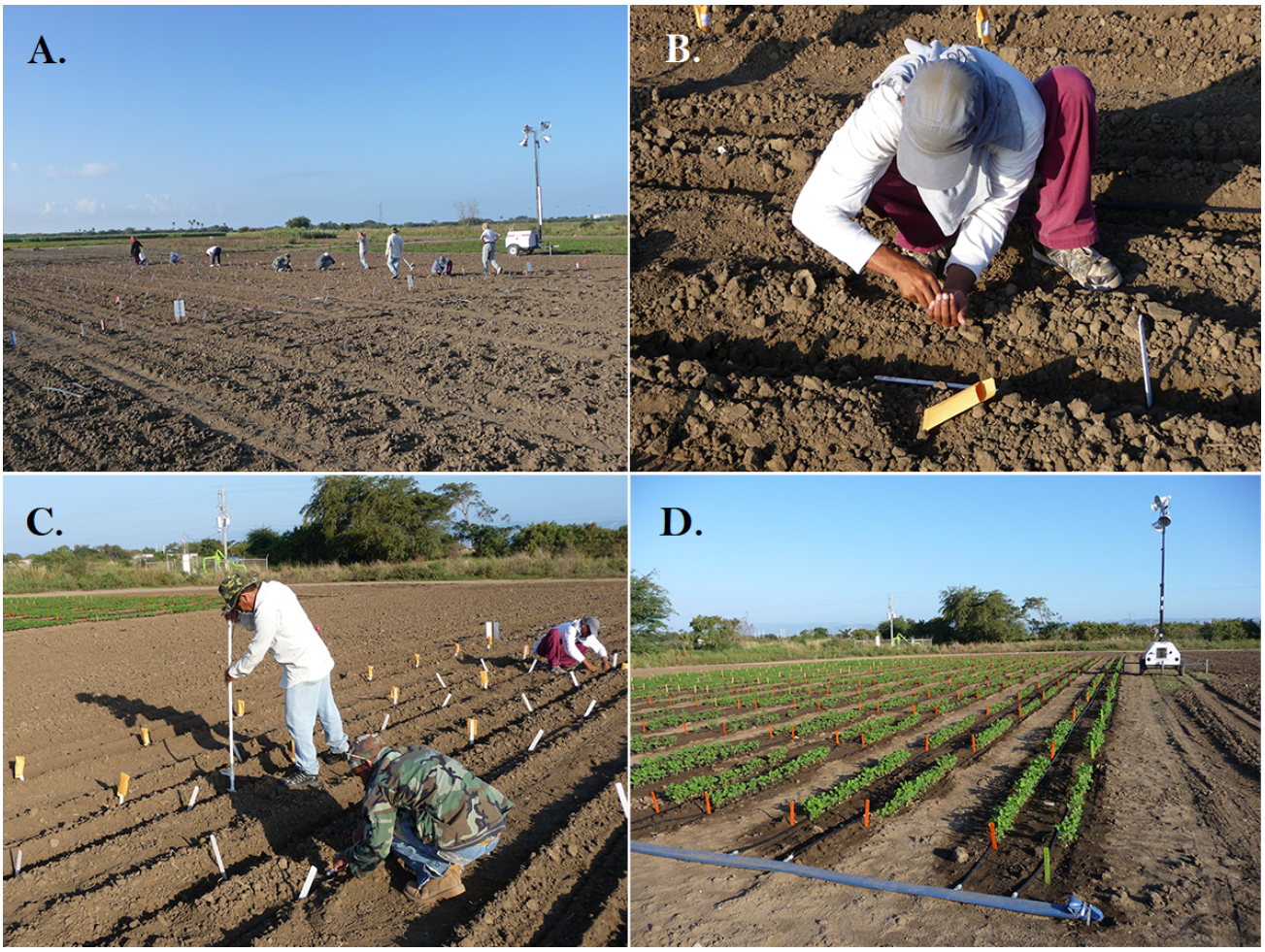


Figure 3. **A.** The hybrid seed is planted by hand in Puerto Rico. The mobile lights on the right are used to extend the day length. Soybeans are a short-day plant and flower when the day length is shorter than a critical threshold. In Puerto Rico, the day length is always shorter than the critical threshold; therefore, the plants adapted to Iowa flower less than a month after planting, unless the day length is artificially extended. **B.** Planting hybrid seed in Puerto Rico. The seed is planted 6 inches apart to obtain as much seed as possible from each individual. **C**. The person on the left is pressing the soybean seed gently into the soil to make good seed-soil contact for germination. The seed will be covered with about 1 inch of soil, after which the soil will be firmly pressed down over the seed. Each stake represents hybrid seed from a different breeding population. **D**. The soybean nursery is irrigated regularly to promote good growth. The lights are used to extend the day length to promote the development of flowers suitable for crossing and to increase seed production.

Review Questions 1

In the following questions, assume that $\mathbf{S_0 = F_2}$.

1. You are breeding for resistance to a herbicide and to an insect in a diploid species. Resistance to the herbicide is controlled by a single dominant allele (H) at one locus. Insect resistance is controlled by a single recessive allele (i) at a locus independent of the one for herbicide resistance. You cross a parent with the genotype (HHII) to one with the genotype (hhii).

    a. Which of the two parents would you use as the female parent in the cross to be able to distinguish phenotypically the hybrid F_1 plants from those resulting from accidental self-pollinations? Why is the elimination of accidental self-pollinations important in a breeding program?

    b. How could you use a molecular marker to distinguish the hybrid F_1 plants from those resulting from accidental self-pollinations?

    c. Under what circumstances would a phenotypic assay for hybrids versus accidental selfs be a better choice than a molecular assay?

    d. You grow the F_2 population and spray it with the herbicide. What would be the genotypic and phenotypic frequencies of the remaining plants for herbicide resistance and for insect resistance?

    e. You harvest one seed from each of the remaining F_2 plants, grow the F_3 population, and spray it with the herbicide. What would be the genotypic and phenotypic frequencies of the remaining plants for herbicide resistance and for insect resistance?

    f. You harvest the F_3 plants individually and plant a progeny row of each as a $F_{3:4}$ line. You spray the lines with herbicide and infect them with the insect. How would you distinguish between homogeneous lines versus heterogeneous lines for herbicide resistance and insect resistance? What would be the expected frequency of $F_{3:4}$ lines that are homogeneous for herbicide and insect resistance?

2. You are breeding for modified protein content in a diploid species that is controlled by recessive alleles at three independent loci. Conventional varieties have the genotype G1G1G2G2G3G3. A parent line with modified protein content has the genotype g1g1g2g2g3g3. The wild-type and recessive alleles at each locus differ by a single nucleotide. You cross a conventional variety to the parent line with modified protein content.

    a. You self the F_1 plants, grow the F_2 population, and test the plants for each of the three loci with the SNP markers. What would be the frequency of F_2 plants homozygous for the recessive alleles at all of the loci, g1g1g2g2g3g3?

    b. Instead of testing the F_2 plants, you harvest one seed from each and use the harvested F_3 seed to plant the next generation. You harvest one seed from each F_3 plant and plant the F_4 population. What would be the frequency of F_4 plants homozygous for the recessive alleles at all of the loci?

Review Questions 2

1. You want to develop 100 $F_{4:5}$ lines of wheat from a single-cross population. Outline season-by-season how you would develop the lines by each of the following methods. Indicate the number of seeds planted and harvested each generation beginning with F_2 seeds. The number of seeds planted should be realistic for meeting the goal of obtaining 100 $F_{4:5}$ lines. Assume that the germination percentage is 80% and that an average of 60 seeds are produced on each plant.

    a. Single-seed procedure of single-seed descent

    b. Multiple-seed procedure of single-seed descent

    c. Bulk method

    d. Pedigree method

    2. Explain to your research director what factors you would consider in selecting one of the methods for use for inbreeding your population. Include in your explanation a discussion of the genetic and financial considerations that would be important.

Review Questions 3

The use of doubled haploids is becoming popular for developing inbred lines used in hybrids, including canola and maize. Use the assigned readings and online resources to answer the following questions.

1. Outline season-by-season how breeders develop doubled haploids of canola and maize:

2. Why has the use of doubled haploids become so popular in the two crops?

3. Are there any disadvantages of doubled haploids compared to the use of traditional selfing for development of inbred lines?

Recurrent Selection

Walter R. Fehr and Walter P. Suza

Readings:

- Chapter 15: Recurrent Selection [pdf], *Principles of Cultivar Development. Vol. 1: Theory and Technique*, by Walter R. Fehr (Access the full book)
- Chapter 16: Genetic Male Sterility for Population Improvement [pdf], *Principles of Cultivar Development. Vol. 1: Theory and Technique*, by Walter R. Fehr

Introduction

The chapters you have been assigned for this section provide an overview of the methods used for **recurrent selection**. It is important to understand the alternative methods in preparation for the first chapters in Principles of Cultivar Development.

Synthetic cultivars

The most extensive use of recurrent selection is for the development of synthetic cultivars, particularly for forage and turf species. In these species, phenotypic selection alone or in combination with genotypic selection is a common practice. The general principles associated with phenotypic and genotypic selection will be illustrated with two cultivars.

The development of FreedomMR represents an example of the use of only phenotypic selection for cultivar development [JPR 2:205-207 (2008)]. The following steps were used by the breeder. The text in the shaded boxes are comments that are provided to assist in understanding the process and the alternatives that the breeder could have considered. These comments are not from the articles.

Cycle 0 population: The synthetic cultivar Freedom was the cycle 0 population used for development of FreedomMR.

The initial population used for recurrent selection is referred to as the cycle 0. Each subsequent cycle of selection is identified with a consecutive number.

Cycle 1: To develop the cycle 1 population, approximately 10,000 plants of the cycle 0 population were

germinated in the greenhouse where they were exposed to natural infections of powdery mildew.[1]It is common for breeders to evaluate large numbers of individuals each cycle for recurrent phenotypic selection. Each plant is a different genotype. The individual plants (genotypes) are commonly referred to as clones.

About 40% of the plants that were susceptible to the disease were discarded. The remaining 60% of the plants were transplanted to the field. About 8.7% of the plants in the field were discarded because they were susceptible. The remaining resistant plants were allowed to intermate by insect pollination.[2]By allowing only resistant plants to intermate, the frequency of favorable alleles in the cycle 1 population would be greater than if the resistant and susceptible plants had intermated and seed was harvested from only the resistant ones. Details of this principle can be found on pages 174-178 of chapter 15 and is the subject of the homework problem for Lesson 13.

The seed from the resistant plants was harvested in bulk with a combine and represented the cycle 1 population.

The nursery in which selected clones are intermated is referred to as a polycross, as discussed in lesson 4. In a polycross nursery, the breeder has the option of growing a single plot of each plant or clonally propagating each plant into multiple replications. The breeder can choose to harvest the seed from all the clones in bulk or can harvest the clones individually and bulk equal quantities of seed from each. Details on alternative procedures that can be used for a polycross can be found on pages 181-188 of chapter 15.

Cycle 2 to cycle 5: The same procedure used for cycle 1 was repeated for cycles 2 to 5. The percentages of resistant plants selected varied among the cycles.

Final cultivar: The resistant plants from the cycle 5 population were selected further for absence of pubescence. It is assumed that the resistant plants without pubescence were allowed to intermate in a polycross to produce the breeder (Syn 1) seed of the cultivar FreedomMR.

The development of Warrior indiangrass is an example of the combination of phenotypic and genotypic selection [JPR 4:115-122 (2010)].

Cycle 0 population: The cycle 0 population was the cultivar Oto, which had a genetic background that traced to accessions from native prairies in southern Nebraska and eastern Kansas.

It is not unusual for breeders of forage and turfgrass species to obtain parent plants from commercial plantings, including pastures and golf courses.

Cycle 1: The first cycle of selection involved the evaluation of 146 half-sib families.

2.

For species in which hybridization occurs by open pollination through wind or insects, half-sib families are formed in a polycross. For the development of Warrior, at least 146 individual clones from the cycle 0 population would have been planted in a polycross. The breeder has the option of replicating the clones in the polycross, as described on pages 421-422 of chapter 33. The clones intercrossed naturally by wind pollination. The seed harvested from each of the 146 clones in the polycross had a common parent, referred to as the tester. The 146 clones themselves served as the tester because their pollen was the source of the male gametes responsible for fertilization to produce the half-sib seed that was harvested. The subsequent evaluation of the half-sib seed was to determine the general combining ability of the 146 clones for quality and yield.

The evaluation of half-sib families for the first cycle of selection as done in this example is less common that conducting recurrent phenotypic selection first, followed by evaluation of half-sib families in later cycles. By conducting recurrent phenotypic selection first for highly heritable traits, the traits of individual clones evaluated as half-sib families may be superior to those selected from populations in which recurrent phenotypic selection was not performed.

The progeny from each of the 146 clones was planted in two replications in the field and data were collected for quality and yield.

During the time that the field test was underway, each of the 146 clones was grown in the field in what is referred to as a maintenance nursery. If a clone is one selected for future use, the vegetative tissue used for propagation is taken from the plant in the maintenance nursery.

There were 29 of the 146 clones selected based on their performance. Two cuttings from each of the 29 clones were used to plant a polycross with two replications.

By clonally propagating the selected individuals, all of the female and male gametes involved in production of the cycle 1 seed came from selected individuals. This principle is described on pages 106-110 of chapter 8 in *Principles of Cultivar Development, Vol. 1*.

The 29 clones intercrossed by wind pollination, the seed from each clone was harvested separately, and an equal quantity of seed from each clone was bulked to form the cycle 1 population.

Cycle 2: Restricted recurrent phenotypic selection (RRPS) was used to develop the cycle 2 population.

The features of RRPS are described on page 181 of chapter 15 in *Principles of Cultivar Development, Vol. 1*. They include the use of gridding and the intercrossing of only selected individual clones.

a. **Gridding**: In the case of Warrior, there were 53 rows in the field, each of which had 14 plants. Approximately three plants in each row with the best visual characteristics were harvested for yield and other traits. The key principle is that the plants in one row of the grid were not compared to plants in other rows of the grid. After evaluation, 39 of the plants were selected for

crossing.

b. **Intercrossing**: After evaluation, 39 of the plants from the cycle 1 population were grown in a polycross. The seed harvested from the polycross was the cycle 2 population.

Cycle 3: RRPS again was used to obtain the cycle 3 population. The 38 selected plants were grown in a polycross. The seed obtained from the polycross constituted the cultivar Warrior.

Clonal, pure-line, and hybrid cultivars

Recurrent selection for these three cultivars types is used to improve populations from which superior individuals can be selected. Unlike synthetic cultivars, the improved population is not used as a cultivar per se.

The type of recurrent selection used for the three cultivar types is influenced by the feasibility of obtaining self-pollinated seed, the impact of inbreeding depression, the feasibility of obtaining hybrid seed, and the role of combining ability in assessing the genetic potential of an individual.

Clonal cultivars

Recurrent phenotypic selection is the most common method of recurrent selection used for species that are grown commercially as clonal cultivars. Combining ability is not a factor in identifying individuals that will perform well as a clonal cultivar, which minimizes the value of testing half-sib or full-sib families. Inbreeding depression limits the value of testing selfed progeny.

Pure-line cultivars

The use of self-pollinated individuals and their progeny is the most common method of recurrent selection for pure-line cultivars because self-pollinated seed is readily obtained. Recurrent phenotypic selection can be used for quantitative traits with high heritability, if it is feasible to obtain enough hybrid seed for the next cycle of selection when intermating selected individuals. Half-sib selection is possible, if enough seed can be obtained from an individual when crossed to a tester. Genetic male sterility has been used in some cases to facilitate the production of hybrid seed by open pollination for recurrent phenotypic and half-sib selection, as described in chapter 16. Production of hybrid seed when crossing two individuals is a limitation for use of full-sib selection.

Hybrid cultivars

All of the methods of recurrent selection are technically possible for improving populations from which inbred lines are obtained for use in hybrids. A comparison of the methods for genetic gain will be discussed in *Principles of Cultivar Development* under the subject of maximizing genetic gain.

Review Questions 1

1. You have developed a random mated population of maize. In screening the population for resistance to aphid resistance, you find 64% susceptible and 36% resistant individuals. Resistance is controlled by the dominant allele P. Resistant and susceptible plants can be identified without error.

a. What is the frequency of the alleles P and p in the cycle 0 population?

b. You eliminate susceptible individuals from the cycle 0 population after flowering. What would be the genotypic and phenotypic frequencies in the cycle 1 population?

c. If you self-pollinate the resistant S_0 plants in the cycle 1 population, what would be the expected frequency of $S_{0:1}$ lines that are heterogeneous for resistance? What would be the genotypic and phenotypic frequencies for resistance within a heterogeneous line?

2. Assume that you are working with the same population as in question 1, but are able to eliminate susceptible individuals from the cycle 0 population before flowering.
   a. What would be the genotypic and phenotypic frequencies in the cycle 1 population?

   b. If you self-pollinate the resistant S_0 plants in the cycle 1 population, what would be the expected frequency of $S_{0:1}$ lines that are heterogeneous for resistance? What would be the genotypic and phenotypic frequencies for resistance within a heterogeneous line?

Review Questions 2

1. Identify an article in any issue of the *Journal of Plant Registrations* for two plant species, other than those discussed above, in which recurrent selection was used to develop a cultivar or germplasm. Describe season-by-season what was done, beginning with the formation of the cycle 0 population and ending with seed from the last cycle of selection. If you are unsure of some aspects of the procedure that was used by the breeder, provide reasonable assumptions of what might have been done. Be sure to include the reference for each of the two articles.

Key Terms

Asexual reproduction

The propagation of an individual from its somatic tissue.

Backcross population

A population in which one parent contributes 25% of the genes and the recurrent parent contributes 75%.

Bulk method

The bulk method allows natural selection to act and remove undesirable genotypes from the population.

Clonal cultivars

In breeding clonal cultivars, hybridization is made between two clones. A large F1 population from the clones is screened as each F1 plant is unique and different from other F1s. This process is repeated over different crop cycles to identify the superior clone for release as a new cultivar.

Cultivars

A group of plants that have certain genetic, morphological, and physiological features that distinguish them from other groups of plants within a species.

Dioecious

Having male and female reproductive parts on separate plants.

Electrophoresis

A technique which uses electricity to separate molecule fragments according to size so they can be studied.

Elite

A crop line that has many genes for good agronomic traits that result in high yields in a particular environment.

Elite cultivars

Cultivated varieties that are well-suited to the typical environmental conditions of the target production

area. These cultivars possess the necessary alleles for key traits such as end-use quality, agronomic performance, disease resistance, and regional adaptation.

Emasculation

The removal of male reproductive parts of a perfect flower or a monoecious plant to prevent of self-pollination.

Experimental breeding line

A line developed from the cross of selected parental lines, resulting in F1 plants. The F1 plants are allowed to intermate over time to produce offspring with stable, pure-breeding lines. The pure-breeding (experimental) lines are used for evaluating specific traits, testing genetic hypotheses, and forming the basis for developing new cultivars.

Hemizygous

An organism that has a gene copy in only one chromosome in a chromosome pair (i.e., during the transformation process, the transgene will insert into only one chromosome of a pair).

Heterogeneous

A cultivar whose plants are genetically different.

Heterozygous

When the alleles at a locus are different.

Homogeneous

A cultivar whose plants are genetically and phenotypically identical.

Homozygous

When the alleles at a locus are the same.

Inbreeding

The process of mating closely related cultivars, or even self-fertilizing, for several generations to select for higher homozygosity.

Inbreeding depression

Reduced survival and fertility of offspring from parents that are related.

Line

Plants within a species that have the same genetic composition and are genetically pure, (i.e., inbred line). Lines are typically experimental, not agronomically competitive (hence not commercially available), and are used only in plant breeding.

Molecular marker

Variant in the DNA sequence used as an identifier or tag of a particular aspect of phenotype and/or genotype. The inheritance of the molecular marker can be traced from generation to generation.

Monoecious

Having separate male and female reproductive parts in the same plant.

Pedigree method

A breeding method used in the development of both self-pollinated (to develop pure-lines) and cross-pollinated crops (to develop inbreds).

Perfect flowers

Flowers that have both male and female reproductive parts in the same organ.

Plant introductions

The acquisition and distribution of germplasm from one country to another.

Polycross

A population in which selected plants (clones) are grown in an isolated nursery where cross-pollination occurs by wind or insect pollination.

Polymorphism

The presence of two or more variant forms of a specific DNA sequence that can occur among different individuals or populations.

Population

Genetically speaking, a population is a group of individuals that share a common gene pool.

Pure-line cultivars

Cultivars that are developed for self-pollinated species. Self-pollination leads to homozygosity and homogeneity.

Recurrent selection

A breeding method involving the selection of plants followed by intermating among the selected plants to increase the frequency of favorable alleles and decrease the frequency of unfavorable alleles in a population.

Sexual reproduction

When the nucleus of a pollen grain unites with the egg cell in the ovary to produce the embryo of a seed.

Single-cross hybrid

The type of hybrid that is produced when two different inbreds are cross-pollinated.

Single-seed descent

A breeding method used to rapidly advance lines to homozygosity so that selection can be practiced on homozygous lines.

Three-way cross

A population in which two parents each contribute 25% of the genes and the third parent contributes 50%.

About the Authors

About Dr. Walter R. Fehr

Walter R. Fehr is an emeritus Charles F. Curtiss Distinguished Professor of Agriculture and Life Sciences, the highest academic honor at Iowa State University (ISU). He obtained graduate degrees in plant breeding from the University of Minnesota and Iowa State University. From 1967 to 2018, he was a faculty member in the Department of Agronomy at ISU where he taught undergraduate and graduate plant breeding courses and conducted research specializing in soybean breeding and genetics. He served as the major professor for 92 students who obtained MS and PhD degrees and authored 270 articles in refereed scientific journals, three books, and 11 book chapters. As a soybean breeder, he developed more than 200 cultivars and was awarded 28 US patents for development of unique genetic traits related to soybean oil quality.

About Dr. Walter P. Suza

Walter P. Suza is the George Washington Carver Endowed Chair and Adjunct Associate Professor at Iowa State University. His research focuses on the metabolism and physiology of plant sterols. He teaches courses on Genetics and Crop Physiology in the Department of Agronomy. In addition to co-developing courses for the ISU Distance Master's in Plant Breeding Program, Suza also served as the director of Plant Breeding e-Learning in Africa (PBEA) for eight years. With PBEA, Suza helped provide access to open educational resources on topics related to the genetic improvement of crops. Suza has worked in central and southern Africa, including at the World Food Programme, Angola, and the United Nations Children's Fund, Zimbabwe, in the areas of food security assessment and mapping of vulnerable households, drought assessment, and coordination of food aid. He holds a Ph.D. in the plant sciences (with an emphasis in molecular physiology) from the University of Nebraska-Lincoln.