

DOUBLED HAPLOIDS: DERIVATION AND EVALUATION

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## I. INTRODUCTION

The primary purpose for doubling the chromosome numbers in a haploid tall fescue (*Festuca arundinacea* Shreb.) plant is to obtain a pure, fertile line with the genetic characteristics of the "parent" haploid plant. Doubled haploid plants should be the genetic equivalents of highly inbred lines, if no mutations occur during any of the chromosome doubling and plant regeneration steps. Therefore, use of haploids and doubled haploids offers tremendous promise for more rapid genetic improvement of wind-pollinated, highly self-infertile grass plants such as tall fescue.

Genetic improvement of tall fescue by conventional procedures is extremely slow.<sup>1</sup> Because of the need for inbreds (or their equivalent) and the natural barriers to inbreeding in conventional grass breeding approaches, we initiated research toward derivation and use of tall fescue haploids and doubled haploids.<sup>2</sup> The procedures used to derive tall fescue haploids are discussed in Chapter 5 of this book. Some of the haploid lines that displayed visually detectable "markers" and/or specific desirable agronomic characteristics<sup>3</sup> were used in experiments designed to obtain fertile doubled haploid lines with the markers and/or agronomic characteristics that were identified in the "parent" haploid plants.<sup>2</sup>

There is a high probability that a large amount of genetic variability exists among gametes in a wind-pollinated, highly self-infertile species such as tall fescue. Therefore, it would be desirable to examine expression of the genetic code in the form of haploid plants under various environmental conditions to eliminate undesirable ones and to select the most promising ones. This procedure would have the effect of allowing identification of desirable genotypes under various field and controlled stress environments. However, haploid tall fescue plants are male sterile and express only a low percentage of female fertility.<sup>4</sup> The objective of this chapter is to discuss theory and approaches used to develop fertile doubled haploid plants from cytologically verified, gamete-derived haploid plants of tall fescue.

## II. DERIVATION

After field testing a number of the tall fescue haploid lines (see Chapter 5 of this book), the next goal was to obtain fertile doubled haploid lines from some of the haploids. Two approaches were used. They were (1) conventional colchicine treatment, as described by Buckner et al.,<sup>5</sup> and (2) a new tissue culture procedure which involved induced endomitotic divisions followed by regeneration of doubled haploid plants from the cells with the doubled chromosome numbers.<sup>2</sup>

Some of the cytologically verified haploids that were cloned via tillers and evaluated under field conditions (see Chapter 5 of this book) were used in attempts to obtain doubled haploids.<sup>2</sup> Five clumps of each of six different

haploid lines were transferred from the field nursery (near Lexington, KY) to a greenhouse in mid February, 1981, after natural vernalization had occurred.<sup>2</sup> Each clump was put in a separate clay pot. Two clumps of each inbred line were treated with colchicine on March 2. Two more clumps of each of the six haploid lines were treated with colchicine on March 10. The other clump of each of the six haploids served as an untreated control and as the source of somatic tissue for the tissue culture approach to doubling chromosome numbers. After colchicine treatment, all plants, including the untreated controls, were grown at  $18 \pm 2^\circ\text{C}$  in a greenhouse. They received supplemental light from cool white fluorescent lamps at  $75 \mu\text{mol m}^{-2} \text{s}^{-1}$  to provide 16-h daily photoperiods. Long photoperiods in the greenhouse after the natural vernalization (which occurred in the field before mid February) caused plants to develop inflorescences.

### A. Colchicine

Plant growth, preconditioning, and colchicine treatments were very similar to procedures used by Buckner et al.<sup>5</sup> in the doubling of chromosome numbers in the 28-chromosome hybrid of annual ryegrass (*Lolium multiflorum* Lam.)  $\times$  tall fescue. Although we followed the previously described protocols, and the process was repeated with a second set of haploid plants and a fresh source of colchicine, no doubled haploid tall fescue plants were obtained by this method.<sup>2</sup> Essentially the same procedure was repeated the following year and, again, no doubled haploids resulted.

The colchicine-treated plants were grown in the long-day greenhouse and observed for pollen-shedding anthers after the panicles emerged. After the panicles dried, they were harvested to determine whether any seed developed. No pollen shed was observed and no seed developed on any of the colchicine-treated plants. It should be noted, however, that greenhouse conditions might have affected pollen development and that failure to set seed could have been due in part to a limited amount of pollen available from other tall fescue plants in the greenhouse. Nevertheless, even if seed had developed on the colchicine-treated plants, there was a high probability that the seed would have been hybrid, because tall fescue is wind-pollinated and highly self-infertile. This would have been inconsistent with our goal to obtain doubled haploid plants that could be cytologically verified, vegetatively increased (cloned), and evaluated under field and other stress conditions before introducing them into a breeding program as pure lines for certain selected characteristics.

Although the objective was to compare doubled haploids derived by the colchicine procedure with those derived by alternate procedures, we abandoned the colchicine approach.<sup>2</sup> At that point, we decided to pursue a tissue culture approach patterned after a procedure that was successful for tobacco (*Nicotiana tabacum* L.).<sup>6</sup>

## B. Tissue Culture

### 1. Development of Rationale

As in many tissue culture approaches, previous research with tobacco served as the model system.<sup>6</sup> Doubled haploids of tobacco can be regenerated quite easily from somatic tissue taken from fully expanded leaves. In the tobacco model system, midvein tissue is excised from a fully expanded leaf of a cytologically verified haploid plant, surface decontaminated, and placed on a solidified culture medium with a tenfold decrease in the auxin/cytokinin ratio (usually as a tenfold increase in cytokinin concentration).<sup>6</sup> Numerous shoots develop directly on the cultured tissue within 3 to 4 weeks. An important point here is the observation that nearly all of the plants regenerated from midveins of expanding ("young") leaves (from a cytologically verified haploid plant) are haploid, whereas many doubled haploid plants are regenerated from midveins of fully expanded ("aged") leaves of the same haploid "parent" plant.<sup>6</sup>

The developmental stage ("young" vs. "aged") of the somatic tissue explant appears to influence the frequency of endomitotic divisions prior to regeneration of plants. This concept was later discussed at the 1974 International Symposium on Haploids in Higher Plants.<sup>7</sup> There appears to be relationship between regeneration of doubled haploid plants and the fact that nearly all cell divisions had stopped in the fully expanded ("aged") leaf tissue.

Although this procedure is very successful for tobacco, considerable modification was needed for tall fescue. Earlier experiments with tall fescue and its hybrid had already revealed that somatic tissue from fully expanded leaves or stems would not form callus, nor would the tissue regenerate plants.<sup>8</sup> In fact, only "young" somatic tissue with numerous dividing or recently divided cells was capable of forming callus on the nutrient media and with protocols used.<sup>8</sup> In that previous research, abundant callus could be initiated from "young" somatic tissue that was excised from the base of rapidly elongating peduncles and internodes, and the callus could be maintained through repeated subcultures on nutrient medium containing 2 mg of 2,4-D per liter.<sup>8</sup> Numerous shoots could be regenerated by placing the callus on the same basic medium formulation with the 2,4-D concentration decreased to 0.25 mg, or less, per liter. If the shoots were regenerated after one or two subcultures of 3 or 4 weeks of duration, nearly all of the regenerated plants had the same chromosome number as the "parent" plant. However, if there were many subcultures on maintenance medium before regenerating shoots, there were often many somaclonal variants among the regenerated plants. When shoots were regenerated after callus establishment and one prolonged subculture on maintenance medium, some of the regenerated plants had increased chromosome numbers relative to the "parent" plant.<sup>8</sup> These combined results from the earlier tobacco and tall fescue experiments provided the basis for successful derivation of tall fescue doubled haploids via tissue culture.<sup>2</sup>

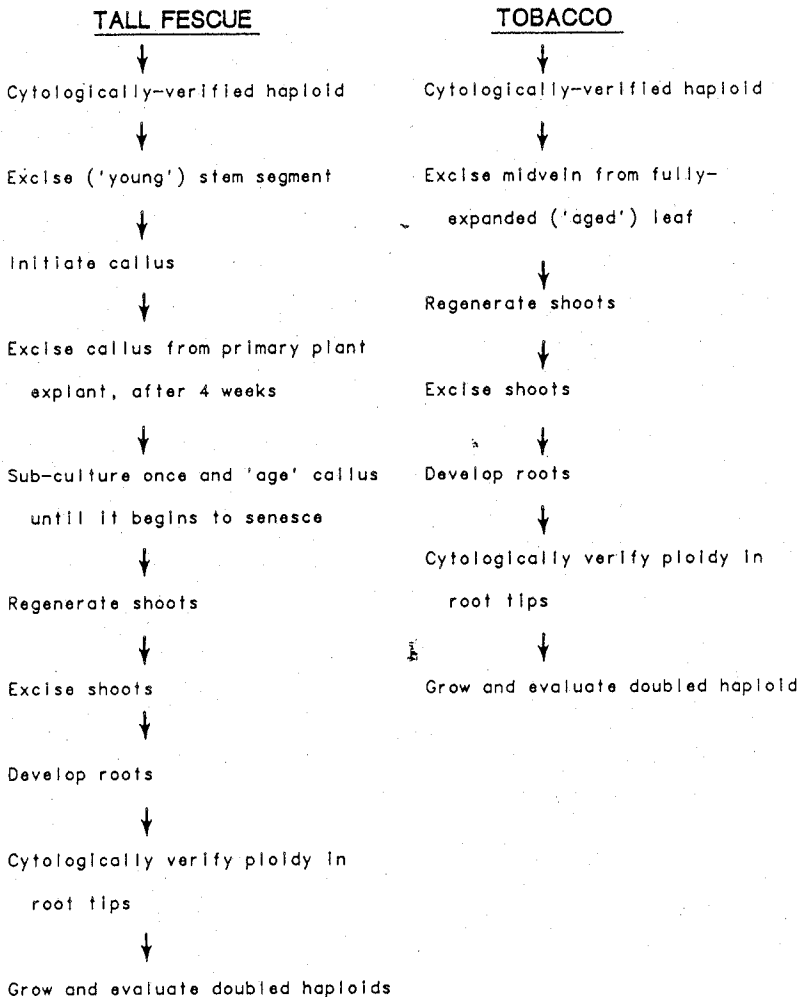


FIGURE 1. Flow chart comparing the regeneration of doubled haploid shoots from somatic tissue excised from cytologically verified tall fescue haploids with procedures used for tobacco. (From Kasperbauer, M. J. and Eizenga, G. C., *Crop Sci.*, 25, 1091, 1985.)

## 2. Procedure

A tentative flow chart (Figure 1) was developed for regenerating doubled haploid tall fescue shoots after considering both the tobacco model system and the previous experiments with tall fescue and its hybrid. The goal was to induce endomitotic divisions in "aged" somatic tissue and then regenerate doubled haploid plants from the cells with the doubled chromosome number. After considering the earlier failure to initiate callus or regenerate plants from

fully expanded ("aged") leaves and stems of tall fescue and the successful regeneration of tobacco doubled haploid shoots from "aged" leaf tissue, we proposed induction of haploid callus from "young" tall fescue stem tissue followed by "aging" the callus before regenerating plants. Further, we were influenced by the previous observation that an increased number of subcultures (prior to regeneration of shoots) would increase the frequency of aneuploids and other somaclonal variants.<sup>8</sup> Considering all of these factors, we hypothesized that the starting point for tall fescue should be establishment of callus from "young" somatic tissue taken from the lower end of rapidly elongating peduncles (or internodes) of cytologically verified haploid plants.<sup>2</sup> We used relatively low levels of sucrose and 2,4-D (20 g and 2 mg/l, respectively) in the callus establishment and maintenance medium because earlier experiments with other grass species produced more albinos and other abnormal plants when higher sucrose concentrations were used.<sup>9,10</sup> Our own experience (Chapter 5 of this book) was consistent with those results.

The soft "young" tissue was excised from the base of rapidly elongating peduncles and internodes of cytologically verified haploid plants that were sampled just as the panicles were beginning to emerge above the flag leaf. The explants were surface decontaminated, cut into 2- to 3-mm segments, and placed on basic callus establishment and maintenance medium (see Table 4 in Chapter 4 of this book) in 20 × 100-mm sterile petri dishes. The cultures were kept in continuous light ( $15 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) from cool white fluorescent lamps in a windowless laboratory that was kept at  $23 \pm 2^\circ\text{C}$ . After about 4 weeks, the callus was excised from the individual explants and transferred to fresh medium of the same formulation (with 2 mg 2,4-D per liter). The relatively small (only a few millimeters in size) pieces of callus were left on the fresh medium for only one extended subculture before transfer to the shoot regeneration (low 2,4-D) medium (see Table 4 in Chapter 4 of this book). The rationale was that rapid callus growth should occur during the first 3 or 4 weeks followed by "aging" of the callus. As predicted, the calli grew rapidly during the first several weeks, then the growth rate slowed and many of the calli had begun to senesce by 15 weeks.<sup>2</sup> At that time, the "aged" calli were subdivided and placed on regeneration medium (the same basic formulation with 2,4-D decreased to 0.25 mg 2,4-D per liter) in petri dishes. The dishes were returned to continuous light ( $15 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) at  $23 \pm 2^\circ\text{C}$  for the regeneration period. Shoots regenerated from the various callus pieces in about a month. When the shoots were about 1 cm tall, they were transferred to rooting medium which contained no 2,4-D and decreased levels of sucrose and agar, as discussed in Chapter 4 of this book.

The mass of callus per initial explant was quite small because the procedure (Figure 1) allowed only one subculture following callus establishment. Therefore, the number of plants regenerated from each haploid plant was relatively low. Somatic chromosome numbers in the plants that were regenerated from "aged" callus from cytologically verified (21-chromosome) haploid tall fescue plants are shown in Table 1.

TABLE 1  
Somatic Chromosome Numbers in Plants Regenerated from  
"Aged" Somatic-Derived Callus Tissue from Cytologically  
Verified (21-Chromosome) Haploid Plants of Tall Fescue

Haploid source plant	No. of regenerated plants	No. of regenerated plants with indicated somatic chromosome numbers					
		21	38	40	41	42	81-84
No. 6	7	6				1	
No. 11	6	5				1	
No. 14	7	5		1		1	
No. 15	19	8		2	1	8	
No. 18	4	2	1			1	
No. 20	17	11				2	4
Total	60	37	1	3	1	14	4

From Kasperbauer, M. J. and Eizenga, G. C., *Crop Sci.*, 25, 1091, 1985.

### III. EVALUATION

After regenerating plantlets from the "aged" callus tissue, they were evaluated in several ways to assess potential value in the tall fescue improvement program. In progressive order, the evaluations consisted of visual examination of the newly regenerated plantlets; cytological examination of shoot and root tips; exposure to various stress conditions in controlled environments; and field evaluation for plant form, winter survival, and fertility.

#### A. Visual

The regenerated plantlets were visually examined at frequent intervals, beginning even before they were transferred from regeneration medium to rooting conditions. The earliest visual examination allowed early identification of green vs. albino plantlets. This allowed determination of the frequency of albinos or other obviously abnormal plant types that may have been associated with various culture conditions and genotypes. The procedure outlined in Figure 1 resulted in very few albino or other abnormal-appearing regenerated shoots.<sup>2</sup>

Visual examinations were continued as the plants progressed through the various stages of regeneration, rooting, early growth, and field evaluation. A few plants developed good leaves but poor root systems, causing them to deteriorate rapidly after transfer to soil. A few other plants had visually detectable abnormalities and were discarded before further time-consuming evaluations. The important point is that tissue selection, culture media, physical environment, and timing of the various steps resulted in many green, normal-appearing plantlets that rooted easily and grew well after transfer to soil.

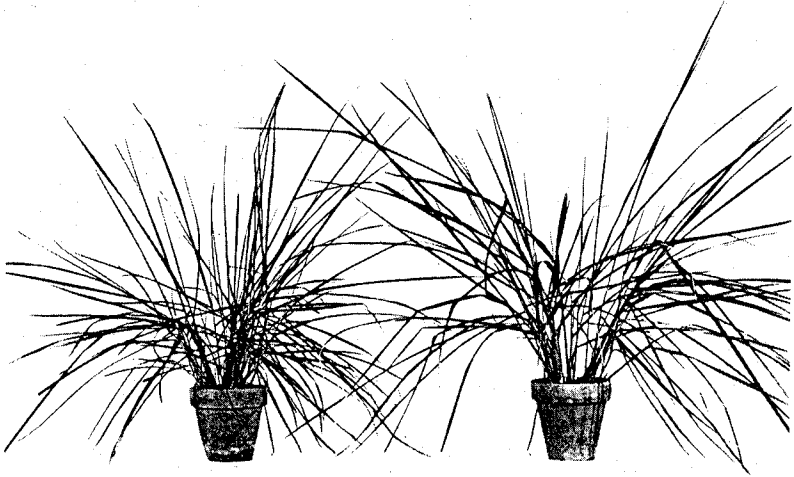


FIGURE 2. An anrogenetic haploid (left) derived via anther-panicle culture and a doubled haploid derived via "aged" somatic tissue culture from the haploid (right). (From Kasperbauer, M. J. and Eizenga, G. C., *Crop Sci.*, 25, 1091, 1985.)

An androgenetic haploid (21-chromosome) tall fescue plant and a doubled haploid (42-chromosome) plant derived via "aged" somatic tissue culture from the haploid are shown in Figure 2. The plants shown in the photograph were each started from a single tiller on the same date and grown under the same controlled environment prior to being photographed. Note that both plants have the same leaf characteristics even though the doubled haploid is larger. This is related to cell size,<sup>11</sup> and the relative size difference between haploid and doubled haploid tall fescue plants is consistent with differences previously observed between tobacco haploids and doubled haploids.<sup>6</sup>

### B. Cytological

The regenerated plants that appeared normal and grew well were examined cytologically to determine whether the tissue culture approach was successful in regenerating doubled haploid plants from the selected cytologically verified haploid plants. There were relatively few regenerated plants because the procedures used to initiate the callus, "age" the callus, and regenerate plantlets were designed to minimize the probability of inducing the mutations that occur with numerous subcultures.

At least one cytologically verified 42-chromosome plant was obtained from each of the 6 selected haploid plants used in this tissue culture procedure (Table 1). The doubled haploid (42-chromosome) plants had the same markers (leaf angle, shape, etc.) as the "parent" haploid plant. Some of the haploids,



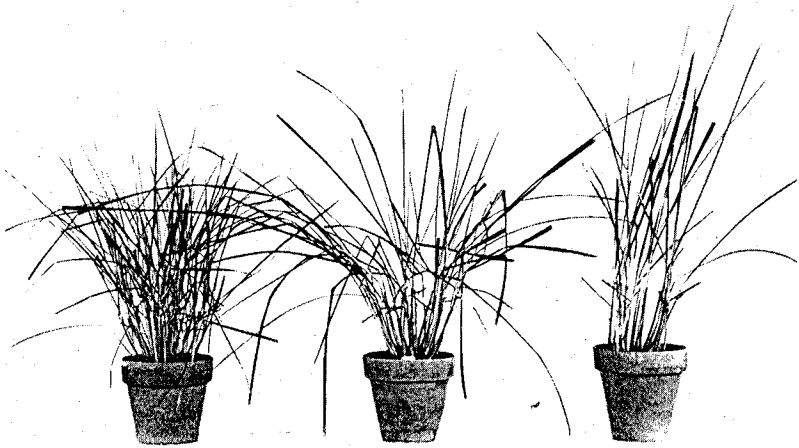


FIGURE 3. Regenerated 21-, 42-, and 84-chromosome plants (left to right) that were derived via somatic tissue cultures from the same cytologically verified 21-chromosome plant (No. 20, see Table 1).

all of the doubled haploids, and one 84-chromosome regenerated plant from this series were cloned via tillering and taken through further evaluations and comparisons.<sup>2,11,12</sup>

### C. Controlled Environment

After cloning, the 21-, 42-, and 84-chromosome plants that were derived from "parent haploid plant no. 20" offered the opportunity to study ploidy effects on various plant characteristics. Representative plants of the three ploidies are shown in Figure 3. All three were started from single tillers on the same date and grown in the same environment prior to being photographed. Leaf size and stem diameter were progressively larger with increased ploidy. These three ploidy levels offered unique material in which to study the influence of ploidy on certain forage-related characteristics (Table 2). Increased ploidy resulted in higher succulence, crude protein, *in vitro* dry matter disappearance, potassium, calcium, and magnesium.<sup>11</sup> However, the 84-chromosome plants were less winterhardy. Also, the potassium concentration resulted in a higher potassium/(calcium + magnesium) equivalent ratio, which has been associated with grass tetany,<sup>11</sup> a physiological disorder of grazing cattle. These preliminary comparisons of ploidy effects on forage characteristics and winterhardiness offer some insight into potential advantages and disadvantages of developing or introducing higher ploidy forage grasses.

TABLE 2  
 Leaf *In-Vitro* Dry Matter Disappearance  
 (IVDMD), Crude Protein, K, Ca, Mg Concentrations\* as Influenced by Ploidy Level in Tall  
 Fescue

	Somatic chromosome no.		
	21	42	84
IVDMD	695 ± 6	715 ± 7	729 ± 5
Crude protein	219 ± 4	227 ± 3	239 ± 8
K	32.2 ± 0.6	34.7 ± 0.9	41.6 ± 0.7
Ca	6.8 ± 0.1	6.6 ± 0.5	7.4 ± 0.2
Mg	3.3 ± 0.1	3.3 ± 0.1	3.6 ± 0.1

\* Values are means ± SE and are given as grams per kilogram of dry matter.

From Kasperbauer, M. J., Karlen, D. L., and Burton, H. R., *Crop Sci.*, 27, 1081, 1987.

#### D. Field

Even though the visual and cytological evaluations indicated that 42-chromosome normal-appearing plants could be cultured from cytologically verified 21-chromosome haploid plants, the final test was that of fertility under field conditions. To test the fertility of the doubled haploid plants, some were grown in field plots surrounded by seed-derived "Kentucky 31" tall fescue plants. Seeds were collected from a number of the doubled haploid lines and from some of the control (Kentucky 31) plants. Randomly selected seeds from such collections were tested for germination on water-moistened filter paper in petri dishes. The seed collected from the doubled haploid plants germinated in percentages as high or higher than seed collected from control plants. When the radicles were about 1 cm long, the seedlings were transferred to individual pots of potting soil in a growth chamber along with control plants. The seedlings from the tissue culture-regenerated doubled haploids grew rapidly and tillered normally in the growth chamber. At this time (1989), many seeds that were collected from the regenerated doubled haploid plants are in cold storage awaiting field trials to compare the progeny of the doubled haploids with conventionally derived plants. The evaluations (both cytological and agronomic) should provide invaluable information on the feasibility of using these biotechnologically derived materials in conjunction with conventionally derived materials in a tall fescue improvement program.

#### IV. CONCLUDING REMARKS

Cytologically verified haploid plants<sup>3</sup> of tall fescue were used in several approaches to the development of fertile doubled haploid plants. Colchicine

treatments were used as suggested by previous literature.<sup>5</sup> However, none of the attempts with colchicine resulted in any doubled haploid plants.<sup>2</sup>

The second approach was a major revision<sup>2</sup> of a procedure that was previously developed for tobacco.<sup>6</sup> With tobacco, it is possible to regenerate doubled haploid plants directly from excised midveins of fully expanded ("aged") leaves of a cytologically verified haploid plant.<sup>6</sup> When such haploid tobacco tissue is placed on a culture medium with a tenfold increase in kinetin, some endomitotic divisions occur and are followed by regeneration of some plants with the doubled chromosome number.<sup>6</sup> Major adaptations were needed before success could be attained with tall fescue. Two major differences were that fully expanded leaf midveins of tall fescue would not form callus or regenerate plants, and young tall fescue tissue did not respond to kinetin in the culture medium.<sup>8</sup>

The successful approach with tall fescue<sup>2</sup> consisted of (1) initiating callus from "young" stem tissue from rapidly elongating peduncles of cytologically verified haploid plants, (2) placing the new callus on fresh maintenance medium for one prolonged (15-week) subculture to "age" it (analogous to the "aging" tobacco leaf<sup>6</sup>), and (3) placing the "aged" callus tissue (in which the rate of cell division had slowed considerably) on plant regeneration medium. Since tall fescue callus appears to be autonomous for cytokinin, the decreased auxin/cytokinin ratio was accomplished by decreasing auxin content in the tissue culture medium.

The plants that were regenerated from cytologically verified (21-chromosome) haploids of tall fescue included some with 21, some with 42, and a few with 84 chromosomes. The three ploidies from a given haploid "parent" plant were useful in evaluation of ploidy effects on various forage characteristics. However, the major findings were that (1) the 42-chromosome (doubled haploid) plants had the same plant "markers" as the 21-chromosome "parent" haploid plants, and (2) that the doubled haploid plants were fertile and produced abundant viable seed when grown in field plots.

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