Project Number: 89-M2

1989-90 Comprehensive Project Report to Almond Board of California

Project Title: Almond Variety Development

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Location: University of California at Davis

Objectives - Long Range:

I. Develop pollenizers for current varieties, particularly Nonpareil.

II. Develop replacement varieties for Nonpareil and other market types that are self-fertile and with a range of bloom time and maturity.

Objectives - 1989-1990:

A) Increase the number of new crosses in the breeding program using low Bud Failure potential material.
B) Better characterize the action of the genes controlling self-incompatibility in almond.
C) Determine the effect of self-fertilization on subsequent kernel size and quality.
D) Develop methods for the genetic transformation of almond and its close relatives.
E) Consolidate, interpret, and publish findings from previous work.

Progress Report- January 1989 - January, 1990:

The breeding of improved almond varieties adapted to California growing conditions has been greatly hampered by severe and persistent problems with Non-infectious Bud Failure since the release in 1938 of the highly susceptible variety 'Jordanolo'. Progress in the understanding and management of this genetic affliction now allows the development of an aggressive and sustained breeding program with the goal of improved pollenizer varieties for Nonpareil, and ultimately, improved, self-fruitful replacements for current varieties including Nonpareil. This past 1989-90 season was the start of a 5 year program to establish a focused variety breeding program utilizing both

proven traditional methods as well as more recent genetic engineering biotechnologies towards attaining these goals. A summary of the breeding strategy is presented in flow-chart form in Fig. 1. The strategy can be separated into 3 basic components:

a) consolidation of a comprehensive and accurate information base for almond production needs and options,

b) establishment of the capability for efficient genetic recombination and genetic manipulation through controlled crosses, and,

c) development of methods for the genetic transformation of established almond varieties, and the identification, isolation, and engineering of novel genes (such as a self-fertility gene) with major value to almond.

Consolidate information base

The varieties 'Padre', 'Solano', and 'Sonora' released by this program in 1984 resulted from crosses and selections initiated before 1965. The development and evaluation of a new variety is a long term endeavor lasting from 10 to 20 years. The current breeding program thus must address not only current problems but future production constraints as well. Our understanding of almond production systems needs to be as accurate, comprehensive, and integrated as possible. For example, if current dormant sprays were lost to the industry, would other controls such as sanitation and/or predatory insects alone be adequate to control targeted pests, or would some improved levels of genetic resistance/tolerance be necessary as well? Does such genetic resistance exist and how difficult would it be to incorporate it into a horticulturally acceptable variety? Might secondary problems such as San Jose Scale, or Aspergillus contamination, which presently are not major limitations to production become so? What cultural and genetic control options might be available? We have made progress in the area of information assimilation with the development of a database for almond knowledge with an emphasis on genetic options, but including cultural, and integrated pest management practices as well. Almond information has been, and continues to be compiled from University of California almond breeding, Cooperative Extension, Integrated Pest Management (IPM) programs, and Sustainable Agriculture Research and Education Program (SAREP) projects. A comprehensive review of the genetic resources available to almond improvement programs is near completion and should be published within the year (1). A review of breeding strategies for almond improvement is underway and should be finished by early summer, 1990 for publication in 1991 (2). Database management techniques allowing an intuitive and integrated analysis and communication

of almond production and management information are now being evaluated for possible implementation during the 1990-91 season.

Genetic manipulation

Crop breeding involves the manipulation of the genetic controls of plant performance in order to optimize desired characteristics including yield and crop value. Two general approaches are presently available for such genetic manipulation; Genetic Engineering, and Genetic Recombination through controlled crosses. Genetic engineering offers the opportunity to add single discrete improvements to a specific variety. This is very useful in that it can complement a serious deficiency in an otherwise excellent variety. Since it adds only a single limited change to a usually well tested and proven variety, subsequent needs for regional variety testing are also reduced. As a result of public anxiety concerning such gene splicing technologies, however, current APHIS regulations mandate fairly rigorous evaluation procedures which negate these potential time reductions. A further disadvantage is that since the improved variety is identical to the old variety except for the newly introduced gene, genetic homogeneity and so vulnerabilities to disease, insect, and environmental problems are increased. Where improvements are desired for multiple traits and/or where the trait is a complex one such as yield and so controlled by several characteristics, Genetic Recombination using controlled crosses or hybridizations remains the only effective strategy. In the almond variety development program we are developing complementary projects in both areas.

Genetic recombination through controlled crosses

Cultivar improvement using controlled crosses involves a reshuffling of the genetic deck and the dealing of new hands. The decks to be shuffled are the parents to be crossed, while the new hands are the resultant progeny. The probability of recovering an exceptional progeny can be optimized by (a) the careful selection of the parents for presence of desired attributes, absence of serious deficiencies and overall complementation or combining ability, and (b) generating large numbers of progeny. Since the entire plant genome of tens of thousands of genes controlling thousands of individual traits is to be recombined, effective parent selection and progeny screening depends upon an accurate understanding and prioritization of both variety needs and available plant genetic resources. The previously discussed information base is being developed to address these needs. New data is also being accumulated through the use of test crosses (crosses made on a relatively small scale to gain information on parent line value).

This last season we have performed several hundred test crosses to obtain information on the mechanisms for self-fruitfulness and self-incompatibility (discussed in the next section), the identification and initial characterization of the 4th cross-incompatibility group in 'Nonpareil' x 'Mission' progeny, and Bud Failure potential and general combining ability between the different parental lines.

Identification of the fourth cross-incompatibility group

Self- and cross-incompatibility in almond is determined by a single gene (designated-S). Since almond is a diploid organism, each individual possesses 2 copies (or alleles) of this S gene (which are rarely identical or of the same allelic types in individual California varieties). The S alleles in Nonpareil can be thought of being S1S2, while those in Mission can be designated S3S4. Since the plant gametes (pollen and egg) receive only one S copy, and normally with equal probability of acquiring either S form, four classes of progeny in equal proportions would be expected from crosses of Nonpareil x Mission as shown in Fig. 2. Almond is self-incompatible since the S alleles in the pollen and flower pistil of the same plant or variety are identical -leading to self recognition and pollen tube abortion in the pistil. Similarly, varieties shown within the same progeny group in Fig. 2 possess identical S alleles resulting in cross-incompatibility through a similar 'self-recognition' response. Knowledge of cross-incompatibility groupings is thus essential to determine effective pollinizer combinations.
Probably 90% or more of the current major almond varieties in California are progeny of crosses between 'Nonpareil' and 'Mission', making this grouping of particular importance. However, while 3 of the 4 groupings have been known for some time, the 4th group had not been identified. Several years of work has now resulted in the tentative identification of this group. Test crosses were designed and carried out which would give progeny segregating for either the S2S3 or the putative 4th (S2S4) group. These progeny were then tested against standard varieties of known cross-incompatibility grouping. Lines belonging to the 4th group can be identified from data summarized in Table 1 since these would be the only lines with different S-S- groupings from all the known standards (and so the only items capable of setting seed in crosses with <u>all</u> of the identified cross-incompatibility groups. The conclusion that 1-98 belongs to the 4th cross-incompatibility group (S2S4) has been further verified though additional test crosses (data available though not included). The identification of the last cross-incompatibility grouping in progeny from Nonpareil x Mission crosses will greatly facilitate the classification of presently unknown varieties since we now have a full set of standards to test against. For example, initial crosses between 1-98 (as

pollen) and the unclassified variety 'Butte' set seed indicating that Butte and 1-98 belong to different groups. Crosses between the unclassified variety 'Monterrey' and 1-98 failed to set seed suggesting self-recognition of identical S allele types and so placement in the same (4th) group. If 'Monterrey' is confirmed to belong to the 4th or 1-98 group in this coming seasons's tests, then varieties in all the other known groups can be expected to be cross-compatible and so effective in pollenizer combinations with 'Monterrey'. As progeny would be expected to segregate to the different groupings with equal probability, several of the presently ungrouped varieties might be expected to belong to this group. Similarly, deviations from this expectation would provide valuable insight into the mechanism determining self-and cross-incompatibility (and so pollinizer efficacy).

Several hundred test crosses to Nonpareil and early flowering peach were made to evaluate Bud Failure Potential in variety sub-clones. Progeny from crosses to an early flowering peach, which show rapid symptom development during the first few years of growth posses an inherent high Bud Failure potential. Similar test crosses to a high Bud Failure potential Nonpareil provides information on the predisposition of individual plants within the sub-clone to this affliction. Approximately 2,400 seedlings from such test crosses are now being prepared for field plantings. Although tedious and requiring several years for final results, these tests provide badly needed qualitative and quantitative information concerning this most crucial almond disease. Several hundred crosses have been made in the spring, 1989 using parents selected for complementation or combining ability based on available records. Such selected combinations are at best, educated guesses, with the true test of combining ability being the performance of the progeny population. Over 2,000 seedlings resulting such these crosses are now being prepared for field transplantation and evaluation this spring.

Large scale crossings

Once good parental combinations are identified very large numbers of progeny are needed to ensure recovery of optimal recombinants. Controlled crosses are currently obtained by tedious and inefficient hand pollinations on previously bagged branches. Successful seed sets of 25-30% are common in good years with much lower sets frequent when the flowering season coincides with poor weather conditions (too wet, windy, hot, etc.). We are experimenting with the use of both honeybees and alternative insect pollinators for controlled pollinations. Small 4 framed hives of honeybees were placed into whole tree enclosures fabricated from surplus parachute cloth and nylon mesh. The donor pollen branches and water were replenished every 3 to 4 days. Adequate seed were set following crosses (selfing would be

Table 1. Summary of seed set on two candidates for the 4th incompatibility group following test crosses to known cross-incompatibility groups as standards.

Standard	Group	X-97	X-98
Nonpareil	S1S2	+	+
Mission	S3S4	+	+
Carmel	S1S3	+	+
Thompson	S1S4	+	+
Ne Plus Ultra	S2S3	-	+
(4th group)	S2S4	(+)	(-)
Butte	?	+	+
Monterrey	?	+	-

postulated S grouping: 1-97 -> S2S3 Ne Plus Ultra
1-98 -> S2S4 (4th group)

prohibited by the self-incompatibility mechanism) in two such test tree enclosures this last season. This experience has also identified the need for better hive vigor control and donor pollen availability in future work. In addition, although the parachute cloth held up well against heavy winds and rain, greater ventilation with nylon mesh fabric was indicated and will be incorporated in further tests this spring. Sarcophagid fly populations were reared on artificial media for testing as a possible alternative pollinator for controlled crosses. This insect was selected since it is easily reared, somewhat gentle (stingless), tolerant to cold temperatures and easily manages in small numbers. For pollination attempts a handful of sedated (by chilling) flies were placed into a small bag containing dehiscing flowers of the pollen donor parent. The resultant pollen dusted flies were then released into a nylon mesh bag enclosing a flowering seed parent branch. Although pollen could easily be detected on treated flies, and the insects proved to be very hardy under field conditions, very low seed set was recovered. This was probably due to a low visitation of open flowers by these flies since most observations found them sunning on vegetative buds or the nylon bag. An additional setback occurred when insects in the first instar of development (=maggots) escaped from their feeding container and invaded the lab. Again these insects proved hardy and resourceful, but the situation was brought under control in only a few weeks.
The evaluation of other insects as pollinators (solitary bees, ladybugs and serphid flies) is continueing.

Screening & Selection

Efficient screening and selection procedures are necessary in order to identify rare superior recombinants from large progeny populations. Screening procedures need to be developed for several important traits, and the intensity of selection for each traits needs to determined. As previously discussed sensitive screening procedures for Noninfectious Bud Failure have been developed to test parent lines. A more rapid and direct (biochemical) procedure is needed, however, to select against this devastating affliction in large segregating progeny populations. Promising procedures for selecting good production capability, based on growth and bearing habit, and kernel and shell characteristics, have been previously developed in this program and are being used extensively.

Evaluations and replicated trials

Evaluation blocks have been planted in Davis and Winters. Future evaluation blocks will be placed at Winters to avoid the excessive bird damage encountered at Davis. Promising selections will also be included in new plantings at the Regional Variety Trials.

Tree performance information is being obtained from the most recent group of almond seedling selections made in 1986. Nursery trees propagated in 1987 were planted in an evaluation block at Wolfskill Experimental Orchards in the spring of 1988. Flowering on some selections in 1989 indicated precocity in certain breeding lines, particularly those with peach or Prunus webbii background. Flower bud formation is apparent in some trees in the 1989-90 winter, and we can expect limited flower and nut production in some trees during 1990.

Genetic Engineering

Genetic engineering and associated biotechnologies offer opportunities for rapid almond variety improvement through the addition of a single high value gene to complement a deficiency in an otherwise excellent variety. Extensive research in genetic engineering and transformation biotechnologies is presently underway, nationally and internationally, in plant, animal, and microbial systems. The goal of our limited work in this area is to anticipate the progress in this field and develop specific materials and techniques to allow the fullest exploitation of these advances for the improvement of almond and peach. At present application of the new biotechnologies to almond is prohibited by:

a) the failure of present genetic transformation procedures with almond, particularly with established varieties such as Nonpareil. and,
b) a lack of engineered genes which would be of significant value to California almonds.

Transformation methods

The predominant plant transformation strategy at present is (a) the use of Agrobacterium tumefasciens to insert the engineered gene into plant cell callus, and then, (b) to regenerate whole plants from this transformed callus. Almond is naturally susceptible to Agrobacterium, so this vector could probably be adapted to achieve transformation of almond tissue. However, whole plant regeneration can only be achieved from juvenile callus (i.e. from seedling tissue, not from mature tissue from established varieties). We are thus researching approaches for the direct transformation of the growing point cells of established varieties. Such an approach would eliminate the need for plant regeneration capability since we are using whole plant material from the start. It also reduces the possibility of deleterious genetic mutations which commonly occur during callus culture. The short term objective of this first season is the design, construction and testing of a low cost (less than

$5,000), efficient device for the high velocity injection of engineered genes into intact plant cells. This work is jointly funded by the California Almond Board and the California Cling Peach Advisory Board at $3,500 apiece, with additional funds of approximately $10,000 from state and federal sources. The design and operation of our present 'particle gun' is described in Fig. 3. Initial tests indicate it is capable of inserting engineered genes into apical meristem or growing point cells of test species (3), and with recent improvements, into almond (Nonpareil) as well (slides available). Work is continueing towards the following goals:

a) demonstrate stable incorporation and expression of inserted genes in Nonpareil and other almond varieties,
b) increase the efficiency of the transformation process to reduce the large time and space requirements for screening out rare transformants,(which would otherwise be too excessive for effective tree crop screening),
c) to reduce the trauma to apical meristem cells resulting from the excision of leaf primordia while exposing the meristem,
d) develop and test engineered genes of value to almond.

Engineered genes

We are continueing to evaluate two engineerable genes with possible value to almond; the Bacillus thuringiensis (BT) gene, and a gene which would confer self-compatibility to established almond varieties including Nonpareil.

Bacillus thuringiensis (BT)

Several types of BT genes have been developed and patented by different biotech programs (Monsanto, Calgene, ect.). Like its bio-insecticide counterpart (Dipel, ect.) BT produces compounds which are toxic to certain insects, mostly Lepidoptera (Navel Orange Worm, Peach Twig Borer, etc.). Initial tests of at least one form of BT inserted into seedling walnut tissue was found to give insufficient protection against Navel Orange Worm (Gale McGranahan, UCD Walnut geneticist/breeder). Further tests of this and other BT genes are planned by both walnut and almond breeding programs. Questions concerning targeted insects ability to breakdown BT toxicity also need to be examined, particularly since it is anticipated that huge areas of agricultural production (for example, tomato and cotton plants in the Central Valley) will depend on similar or even identical BT genes for protection. Reports of the breakdown of BT protection have already been reported, thought the stability of the bio-insecticide form (Dipel, ect.) over the past decades has also been well documented.

Self-compatibility

A long term goal of this breeding program is the development of a self-fruitful Nonpareil type almond. Earlier work has shown that self-fruitfulness could be partitioned into pollen-pistil compatibility and ability for self-pollination (4). Physiological and morphological studies during this past season have identified two possible mechanisms of self-pollination; the convoluted growth of the flower style into the pollen bearing anther sacs leading to the direct transfer of self pollen, and an indirect mechanism where pollen is first transferred to petals through wind movement and then from the petals to the pistil stigma. Crosses for testing the genetic control of the first mechanism will be initiated in the spring of 1990. Related studies have found that pollen shed in almond, peach, and almond x peach hybrids, is strongly dependent upon relative humidities with considerable variability found between varieties (Weinbaum, unpublished).

The control of self- and cross- incompatibility in almond by a single major gene was supported by the segregation ratios of this last season's 4th incompatibility group studies, as well as earlier published results (xxx). The apparent partial breakdown of this self-incompatibility gene in the variety Jeffries has been studied this past season. Although Jeffries's origin as a mutation of Nonpareil is supported by isozyme and morphological data from this season and previous work (5), Jeffries will set seed with Nonpareil pollen, (though it remains self-incompatible to its own pollen or in the reciprocal cross). Studies on the physiology and genetics (and utility for developing self-compatible lines) will continue this spring.

The existence of self-compatible forms of this (S) gene has been demonstrated by the transfer of self-compatibility from peach (Prunus persica) to several of our almond x peach evaluation lines. Molecular studies by other research programs have concluded the structural similarity between self-incompatibility genes from even distantly related species. Work on the identification and isolation of the S-gene from the related sweet cherry (P. avium) is now taking place in Australia. Successful work at these molecular labs would greatly facilitate the identification, isolation, and cloning (multiplication) of self-fertile S-gene types functional in almond. Rather than attempting to duplicate the expensive and tedious initial isolation, we are developing parallel programs to allow the rapid use of this information when it becomes available towards almond improvement.

Effect of self-fertility on kernel quality and yield

The forced selfing of a normally self-incompatible species often results in weak and aberrant development in progeny, a phenomena known as inbreeding depression. To test whether self fertilization in standard almond varieties would jeopardize kernel shape and size, methods were developed which allowed the forced selfing of Nonpareil, and simulated large scale selfing of Nonpareil by using Jeffries (a sport of Nonpareil, and so genetically almost identical) as seed parent in crosses to Nonpareil. No difference in kernel morphology was observed between actual selfs, simulated selfs, and normal, outcrossed Nonpareil (slides available). Very small though statistically significant differences were detected in similar comparisons of kernel weight (Table 2). These findings indicate little to no detectable loss of kernel quality and weight (and so yield potential) would be expected in self-compatible almond varieties developed either by genetic engineering or controlled cross methods.

Table 2. Seed weight statistics from forced self-fertilizations of 'Nonpareil' and analogs of self-fertilizations of the 'Nonpareil' mutant "Jeffries' and their respective controls.

Cross	Mean	STD	No.
'Nonpareil' outcrossed	1.24	0.121	11
'Nonpareil' selfed	1.25	0.156	11
'Jeffries' outcrossed	1.26	0.134	131
'Jefferies' x 'Nonpareil' (the equvalent of a self)	1.25	0.136	184

Conclusions:

(a) Differences between 'Nonpareil' selfs and outcrosses are small and variable but significant (0.05 level) based on paired t-test.

(b) Differences between 'Jeffries' x 'Nonpareil' which is analogous to 'Jeffries' selfed (since 'Jeffries' is a bud sport of 'Nonpareil') and 'Jeffries' outcrossed are small but significant (0.05 level) based on ANOV test.

(c) Althought statistically marginally significant, these differences are negligible horticulturally.

Conspectus

The Almond Variety Improvement project is a multi-disciplinary, multi-faceted and multi year project. Emphasis is on the implementation of effective strategies towards informed and focused goals. These strategies involve the development of both a sound yet flexible infrastructure for genetic manipulation and a fuller utilization of Federal, State, and private resources in applied and basic research towards the ultimate objective of improved California almonds. Although the Almond Board of California funds constitute only a small portion of the annual funds, this funding source provides the very important seed moneys, both for attracting other grants and research interest and for ensuring the continuity of this long term project.

Citations

1. Kester, D.E., T.M. Gradziel and Ch. Grasselly. Germplasm Resources of the Almond. In J. Ballinger (ed.) Genetic Resources of Fruit and Nut Crops. (Manuscript submitted for inclusion as chapter in text). 70pp.

2. Kester, D.E. and T.M. Gradziel. Almond. In J. Janick and J.N. Moore (eds.) Advances in Fruit Breeding. (Manuscript in preparation for inclusion as chapter in text).

3. J.H. Oard, D. Paige, J. Simmonds and T. Gradziel. Transient gene expression in maize, rice, and wheat cells using an airgun apparatus. (Accepted - Plant Physiology).

4. Weinbaum, S.A., D.V. Shaw and T.T. Muraoka. 1989. Independence of self-compatibility and potentiality for self-pollination in peach x almond hybrids. Euphytica 41:53-58.

5. Hauagge, R., D.E. Kester, S. Arulsekar, D.E. Parfitt and L. Liu. 1987. Isozyme variation among California almond cultivars: II. Cultivar characterization and origins. J. Amer. Soc. Hort. Sci. 112:693-698.

Fig. 1. Flowchart of Breeding Strategy for Almond Variety Improvement

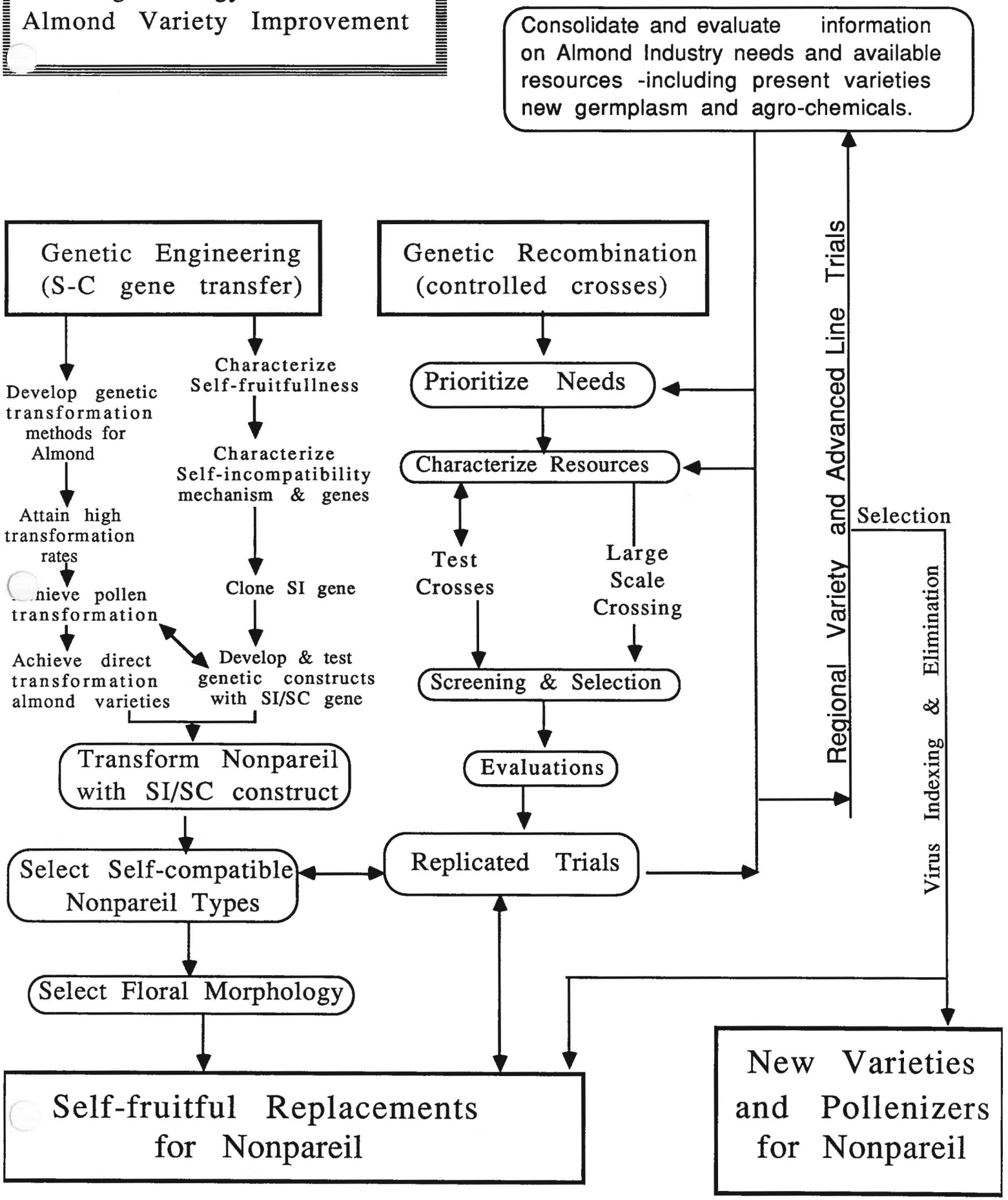
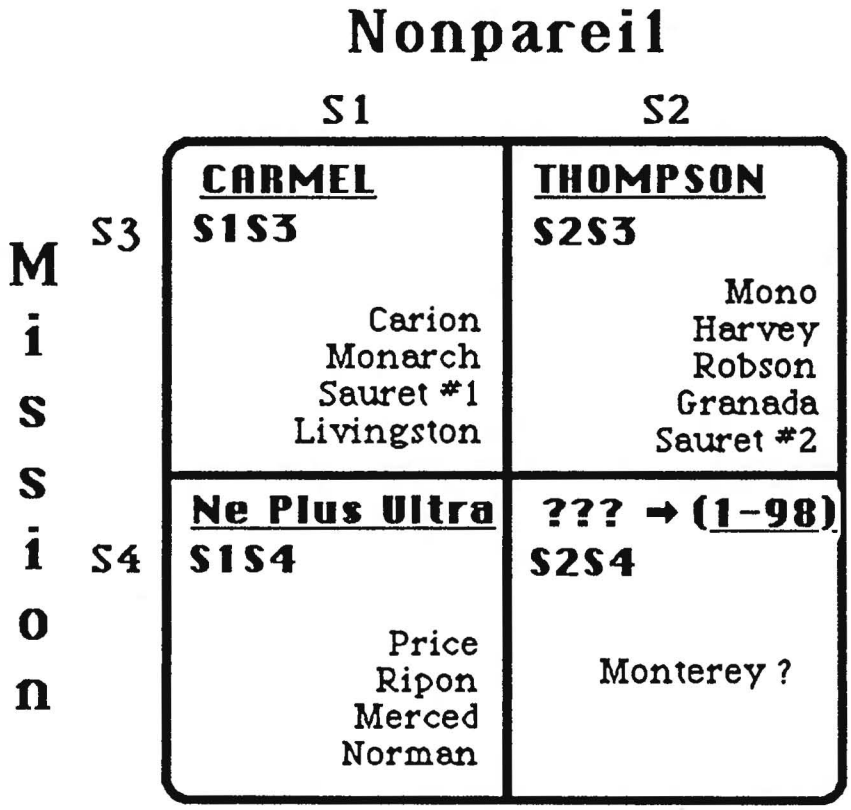


Fig. 2. Pollen Incompatibility Groups in Nonpareil x Mission Progeny



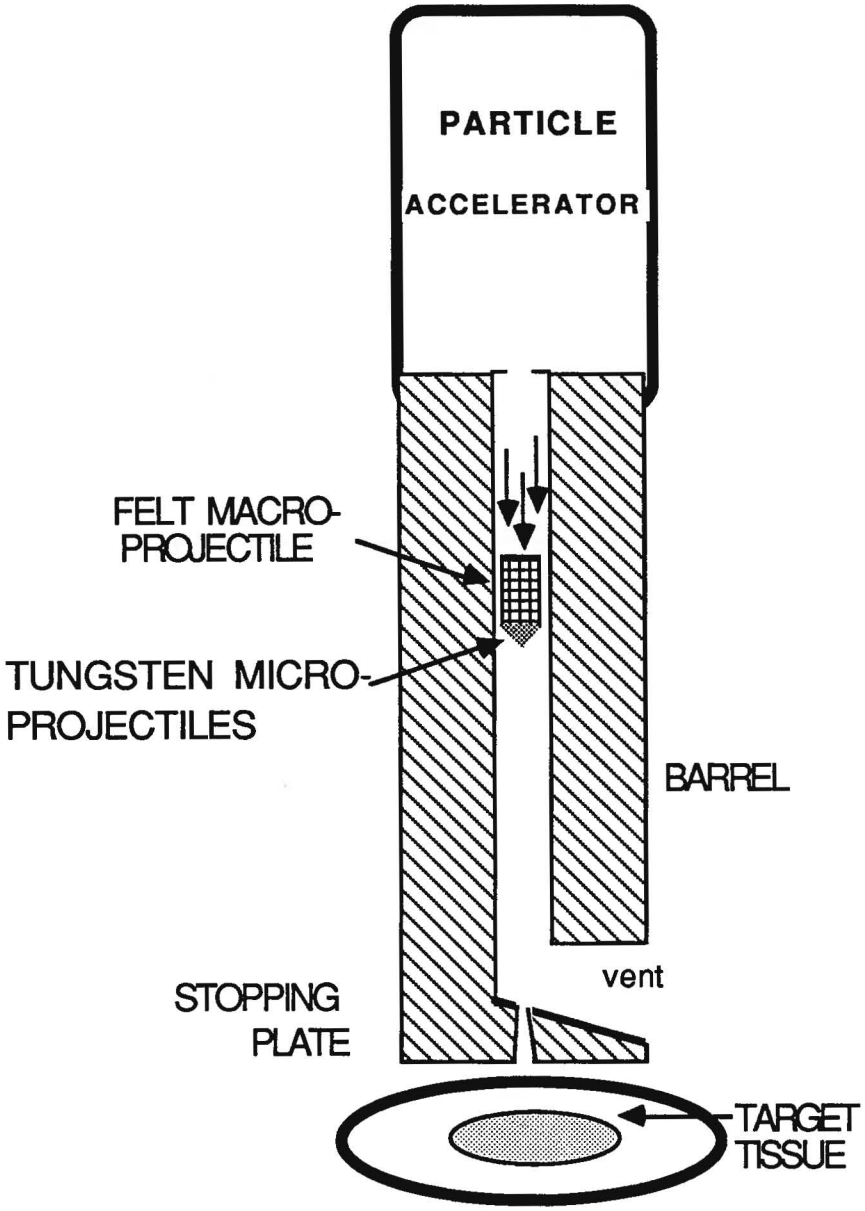


Fig. 3.

Schematic Diagram of Particle Accelerator for Almond Transformation

Approximately 0.05 mg of tungsten particles with diameters of 0.5 um is placed on the front surface of a cylindrical felt projectile (5mm diameter, 8mm length). Engineered DNA can be adsorbed to the tungsten particles using calcium chloride or alchohol precipitation. The macro-projectile is propelled at high velocities into the stopping plate where it is stopped or deflected. A small orifice in the stopping plate allows the micro-projectiles to continue unimpeded into target cells 1 to 5 cm away. Such very small particles at very high velocities have been capable of injecting functional DNA through plant cell walls and membranes in a non-lethal manner.