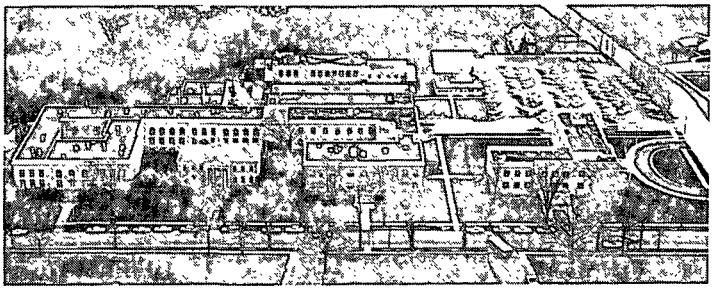

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INTEGRATION OF MOLECULAR AND CLASSICAL GENETICS

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Integration of Molecular and Classical Genetics

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INTEGRATION OF MOLECULAR AND CLASSICAL GENETICS

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Abstract: Tree improvement programs based on classical genetics have yielded significant increases in forest productivity. Several factors nevertheless continue to hamper progress. A variety of techniques from molecular genetics, e.g., gene transfer and in vitro selection, should help increase efficiency and lower costs. Integrating these into classical programs will require efficient means for regenerating seedlings. Somatic embryogenesis holds much promise in these regards, and substantial progress has been made in developing it. Effective integration also requires a balanced mix of research, one that will maintain progress in classical genetics, develop molecular tools, and make available an economical regeneration system.

INTRODUCTION

Recent forestry literature contains much testimony about the new "molecular genetics," and the use of such technologies in improving forest growth, health, and quality. Some authors (e.g., Krugman 1985) argue that technologies ranging from tissue culture through protoplast fusion to "genetic engineering" are poised to revolutionize forest tree breeding. Applied properly, the newer approaches clearly have the potential for expediting classical tree breeding, cheapening the process, and creating better-adapted, faster-growing, and higher quality fiber sources. Given the many difficulties faced by tree breeders, such technologies may well have greater impact in forestry than that now observed or anticipated in agriculture (Dinus 1985). Just how rapidly this "revolution" will unfold and yield practical results nevertheless remains conjectural (Hanover 1987).

Sound support for classical genetics/breeding programs is necessary for continued improvement. Classical programs, after all, remain the stage on which molecular approaches will play, interact, and produce benefits. Care must be taken to ensure that the stage is set properly, and that all needed plant materials, technologies, and players are present. Judicious support is also required to develop appropriate molecular technologies on a timely basis. More important, implementing molecular shortcuts and effectively integrating classical and molecular approaches depend on simple and reliable systems for regenerating plants (Stomp 1988).

My purpose is to review the status of classical genetics programs, describe some parallels with and potential applications of molecular genetics, and address the use of a promising regeneration system, somatic embryogenesis,

as an "integrator" of classical and molecular programs. In keeping with this purpose and the intent of symposium organizers, this paper focuses mainly on research at my home organization, The Institute of Paper Chemistry (IPC). Developments elsewhere, mainly recent ones, are mentioned, but in a far from exhaustive manner. Attention is also focused on conifers, without meaning to infer that progress is lacking in hardwoods. Indeed, some remarkable achievements have been made recently (Fillatti et al. 1986, Merkle and Sommer 1987, Mascarenhas et al. 1988).

CLASSICAL GENETICS

TREE IMPROVEMENT - STATUS, PROCESSES, AND CONSTRAINTS

Classical tree improvement programs have been implemented in many countries, and much progress has been made in a variety of species. Gains in growth, quality, and pest resistance have been substantial in early generations and are expected to be even greater in later ones. Benefit/cost analyses clearly indicate favorable returns on investment, and procedures considered experimental only a decade or two ago are now applied routinely by many industrial and governmental organizations. To hasten implementation and raise efficiency, research in recent years has concentrated on improving seed orchard production and protection, seed collection, handling, and storage, nursery practices, and plantation establishment.

The classical improvement process is perhaps best described as a cycle, with each of several steps repeated across generations (Fig. 1). Selection and breeding are the main tools used by breeders to collect, add, and recombine useful genes. After desirable phenotypes are selected, they often are moved immediately into "production populations" for sexual or asexual production of

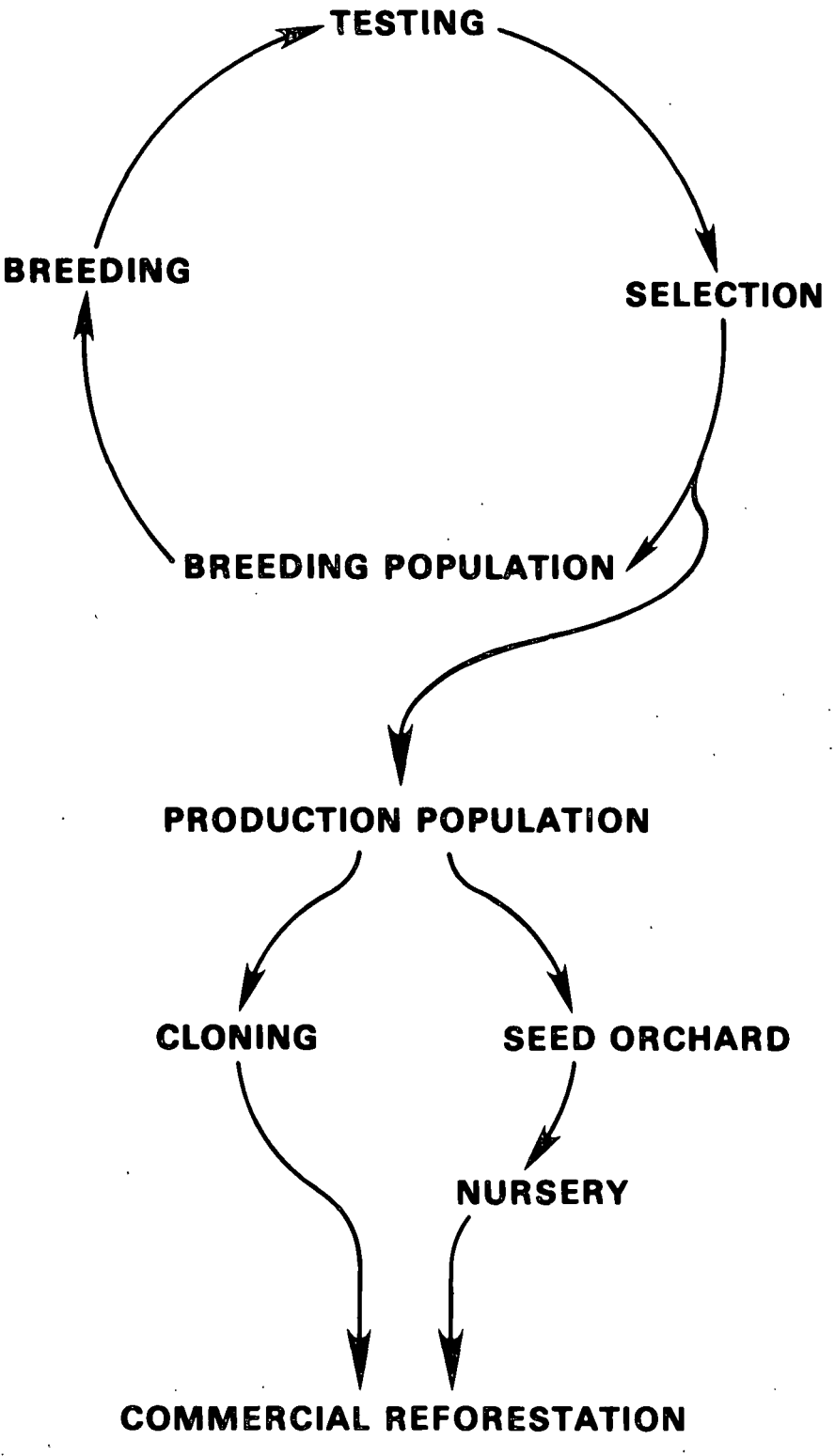

Figure 1. Classical genetics: the tree improvement process.

improved planting materials, thereby hastening application in commercial reforestation.

Simultaneously, selections are merged into "breeding populations" for intermating and creation of new generations. To ensure broad genetic bases and minimize inbreeding, breeding populations are kept large. New selections or individuals from other regions and even other species are sometimes incorporated to add new and different genes. Offspring from the breeding step are established in field tests to verify genotypic worth of original selections and provide material for the next cycle of selection and breeding. Information from such trials is also used to remove less desirable individuals from production populations, further raising the frequencies of useful genes and increasing genetic gain.

Though the process is straightforward and effective, classical tree improvement remains difficult and expensive. Most commercially important tree species are large, have long lifespans, and are slow to attain reproductive maturity. These and other factors limit progress and efficiency.

Selection is complicated by long lifespans. Most important traits must be evaluated at or near harvest age, unless strong correlations exist between juvenile and mature performance. Such correlations are often lacking, and years pass before significant returns are earned on the substantial investments needed to start programs.

Breeding is hampered by large tree size, and especially by delayed reproductive maturity. Saving time, if possible, requires using complicated and expensive techniques to stimulate early flowering and fruiting.

Most important traits are inherited quantitatively, and are much influenced by environmental factors. As a result, extensive field testing, often of expensive control-pollinated offspring, is essential. This step is both costly and time-consuming as evaluations must be made periodically, with final judgment often deferred until or near harvest age. Testing is further complicated by tree size. Sound tests require large numbers of offspring, and therefore large amounts of land and labor. Using small numbers lowers costs but also reduces selection efficiency.

Though not all inclusive, the foregoing serves to highlight some of the factors that have limited and continue to constrain classical tree breeders. Advances in classical genetics have resolved some problems and have provided many shortcuts. Effective integration of classical and molecular genetics could resolve some long-standing problems and open new horizons.

MOLECULAR GENETICS

PARALLELS, TECHNIQUES, AND PROMISES

Numerous parallels exist between classical and molecular genetics. The main tools of classical genetics - selection, breeding, and testing - clearly have counterparts in molecular genetics (Riemenschneider et al. 1988). Some examples of useful molecular approaches (Fig. 2) are in vitro screening, restriction fragment length polymorphisms (RFLP's) and other molecular markers, genetic engineering or directed gene transfer, protoplast fusion, and somaclonal variation and selection.

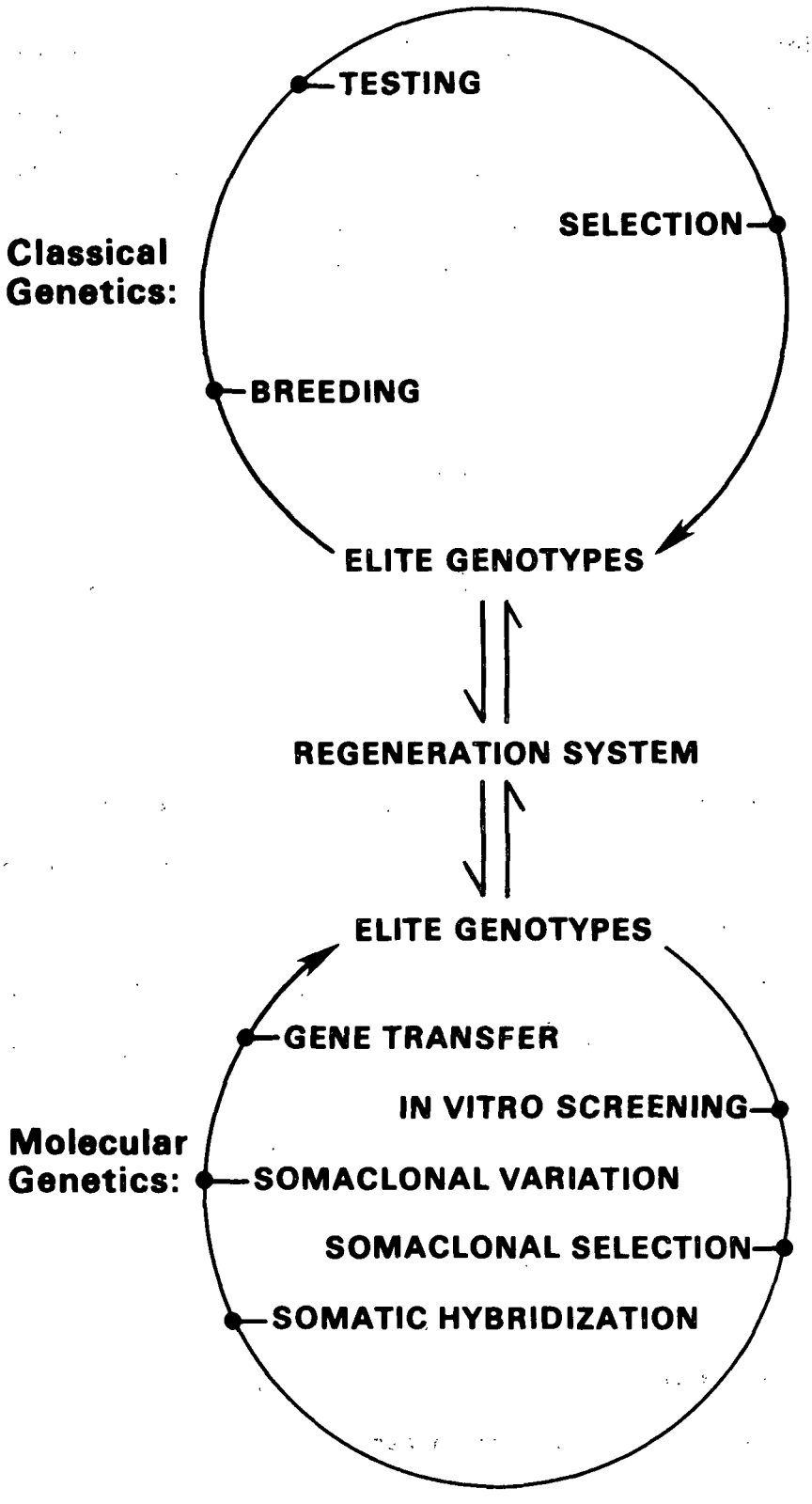

Figure 2. Reliable and efficient regeneration systems are essential for effective integration of classical and molecular approaches.

Selection and testing may be done in cell and tissue culture, provided that the particular trait is expressed in vitro or that a correlation exists between in vitro and field performance. Cells or tissues from thousands of individual genotypes can be exposed to a selective agent or environment in a small laboratory. The best individuals, thus identified, could be moved rapidly into breeding and production populations. Or, given a reliable regeneration system, selected cultures could be used for mass asexual propagation of useful variants for reforestation. Large scale screening would greatly improve selection efficiencies as well as offset disadvantages posed by the long life spans and large size of most tree species. Such techniques have yielded herbicide tolerance (Michler and Haissig 1988) and disease resistance (Ostry and Skilling 1988).

When available, RFLP's and other molecular markers will permit large scale and early screening for presence of a particular gene or combination of genes. Selection for both qualitative and quantitative traits should be possible. Numerous individuals could be evaluated, with only the best few used in further breeding or testing. The ability to work with larger numbers and select at earlier ages than otherwise possible should yield savings in both time and expense.

Classical tree breeders add and recombine useful genes via intra- and/or inter-specific hybridization. Parallel approaches in molecular genetics involve nonsexual addition and recombination via methods such as direct gene transfer, somaclonal variation, and somatic hybridization.

Gene transfer or so-called genetic engineering involves identification, isolation, and movement of useful genes within and among tree species or from

other organisms. Several gene transfer methods, e.g., transformation via Agrobacterium spp., electroporation, and microinjection, have been tested. Decades may pass before routine application, however, in that little is known about "tree genes," rather few useful genes are available from other organisms, systems for regenerating plants are far from reliable, and testing and release procedures are subject to the uncertainties of legislation. Feasibility and potential utility have been demonstrated by transfer of a gene for herbicide tolerance from a bacterium into a Populus hybrid (Fillatti et al. 1986). Such methods bypass the sexual process, thereby avoiding the long time required for most tree species to attain reproductive maturity, and enabling breeders to make rapid changes in direction.

Cell fusion, somaclonal variation, and related techniques also offer opportunities for adding and recombining desirable genes. Creation of dihaploids, as an example, could be useful for genetics research, for improving selection efficiency, and for developing true-breeding hybrids. Protoplast fusion to combine genes from unrelated or incompatible species in special purpose hybrids is another possibility. Moving chloroplast or mitochondrial genes, or even individual chromosomes of contrasting genotypes, represent yet other possibilities.

Predicting just when molecular techniques will routinely complement classical approaches is beyond the scope of this paper. Suffice it to say, however, that some time will pass before most move from the research to the development or application stage. Most likely, individual techniques will be ready at different times, and applied independently. Some argue that application will occur in stepwise fashion, with reliable tissue culture methods and

large scale asexual propagation coming first (Hanover 1987). This seems probable and fitting in that many molecular techniques depend on reliable cell and tissue culture systems for research and development and regeneration of genetically modified plants.

Given such dependencies, efficient regeneration systems are essential for capturing the benefits of molecular genetics as well as for rapidly and thoroughly capitalizing on classical breeding programs. Indeed, they can be viewed as the link or mechanism for effective and efficient integration of the two "genetics" (Fig. 2).

REGENERATION SYSTEMS AS "INTEGRATORS"

Several approaches have been advanced for clonal propagation of forest trees. Macropropagation or rooting of cuttings, is used for some hardwoods (e.g., Eucalyptus spp.) and a few conifers [e.g., Norway spruce (Picea abies)]. The method is also being evaluated in loblolly pine (Pinus taeda) (Foster and Shaw 1987). For many species, however, the process is slow and laborious, is generally applicable to only young trees, and yields low numbers of propagules costing considerably more than sexually derived seedlings. Gene transfer can be effected (Sederoff et al. 1986), but procedures are inefficient and results vary with species. Many useful molecular techniques cannot be exploited.

Micropropagation involves regeneration from preexisting meristems or adventitious buds. This approach is more efficient than macropropagation, and appears economical for use in Eucalyptus spp. (Mascarenhas et al. 1988) and several conifers (Hasnain et al. 1982, Aitken-Christie et al. 1988). For most conifers, however, laborious manipulations and relatively low multiplication rates have kept propagule costs above favorable levels. In addition, the

approach is not well suited for efficient application of many molecular techniques (see Webb et al., these proceedings).

Somatic embrogenesis, a third approach, seems more promising for economical mass propagation of planting materials improved by either classical or molecular means. This approach involves production of embryolike structures, bearing both root and shoot meristems, from cell and/or tissue cultures of somatic tissues. Somatic embryogenesis has been obtained in a variety of conifers. With time and refinement, culture in liquid media should permit large-scale production. Encapsulation to form synthetic seeds (Gray 1987) should permit delivery through conventional forest nursery systems.

The cell and tissue cultures used to obtain embryogenesis can also be used for testing, selection, and direct gene transfer, and will become useful for protoplast fusion and other methods for adding or otherwise altering genetic composition. When developed into an effective regeneration system, embryogenesis should provide the much-needed link between classical and molecular genetics (Fig. 3). Effective and efficient integration thus demands continued research on regeneration systems, and somatic embryogenesis promises to be the most versatile and economic system.

PROGRESS ON SOMATIC EMBRYOGENESIS

Somatic embryogenesis was first obtained in conifers from cultures of immature embryos of Norway spruce (Hakman and von Arnold 1985). Progress has been dramatic since this major breakthrough, and somatic embryogenesis has been reported for 11 other conifers (see Becwar 1988, von Arnold and Hakman 1988a, Webb et al. these proceedings). These many recent reports suggest that the

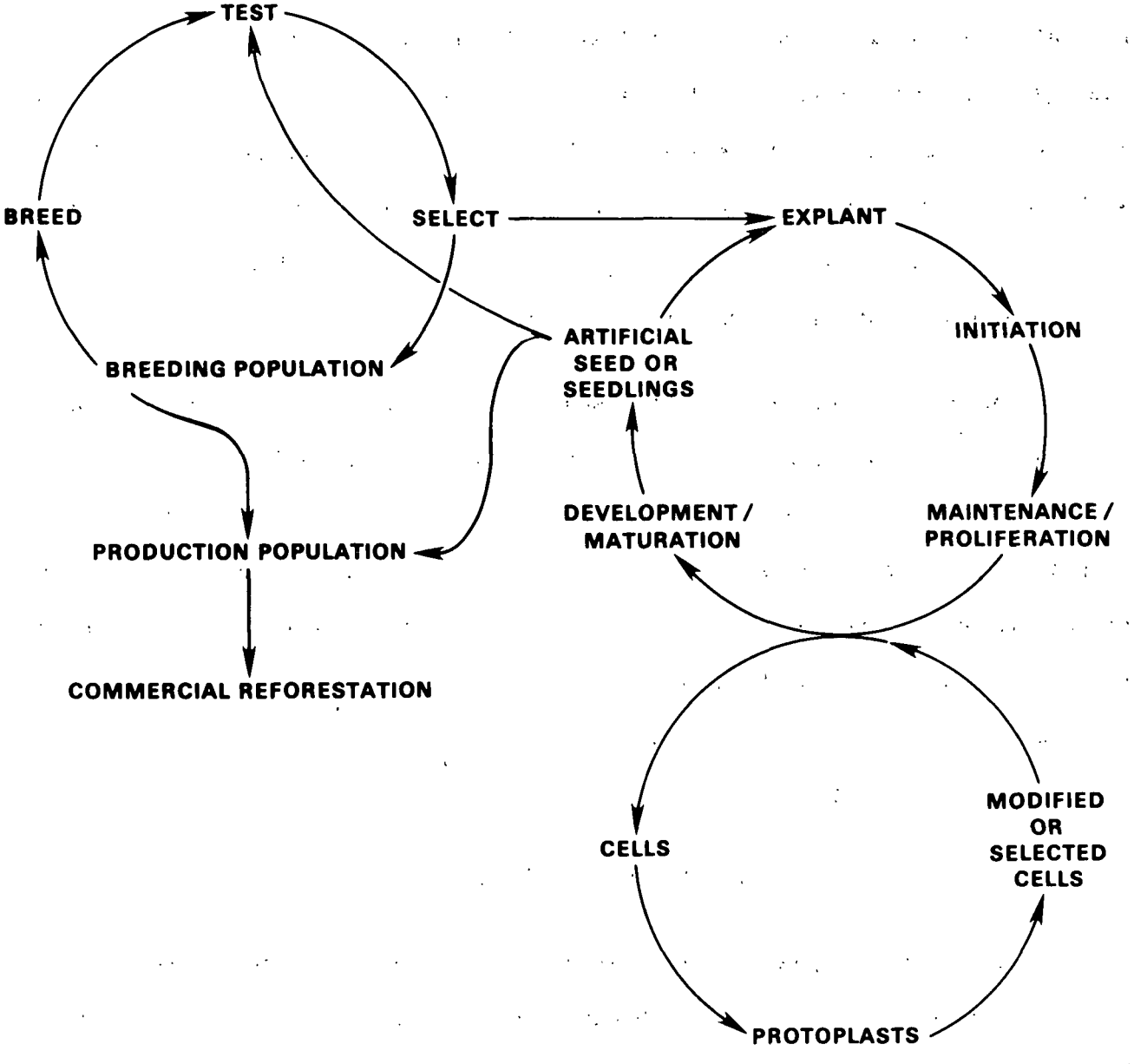

Figure 3. Somatic embryogenesis as an "integrator" of classical and molecular programs.

process will be extended to yet more species as experience is gained and methods are improved.

Seedlings have been recovered from somatic embryos of several conifers, but yields typically are low. Most workers have concentrated on initiation of embryogenic cultures (EC). Because of this focus and low embryo numbers, optimal methods for embryo maturation and other process steps (Fig. 3) have not been developed. As more EC become available, however, other steps will be investigated and undoubtedly improved.

IPC workers have obtained EC in Norway and white spruce (Picea glauca), white (Pinus strobus), pond (P. serotina), and loblolly pines, and, more recently Douglas-fir (Pseudotsuga menziesii) and the hybrid between pitch (Pinus rigida) and loblolly pines. Efforts are concentrated on loblolly pine and Douglas-fir, with Norway spruce used as a model for testing protocols. Much work has also been done on quantifying success rates for individual steps (Becwar et al. 1987a), so as to identify limiting steps and concentrate effort thereon. The goal is to raise efficiencies of critical steps, and the overall process, to levels suitable for regeneration on a commercial scale and for application of molecular techniques. Since few authors stress quantification, IPC data form the bases for much of the following discussion.

EXPLANT

Explant source and developmental stage greatly affect initiation success. In conifers, immature or mature zygotic embryos have been most responsive. Cotyledons from newly germinated seedlings have proven responsive in a few species. For example, EC has been obtained at IPC from cotyledons of 10- to 14-day-old Norway spruce seedlings, but frequencies have been only one or two

percent. Somewhat higher frequencies have been reported by Krogstrup (1986) and Lelu et al. (1987). Such successes mark an important step forward. Until explants from more mature sources can be used routinely, however, application will be restricted to relatively few genotypes and to materials not old enough to have been proven genetically superior.

INITIATION

Regardless of species or explant, EC have the same general phenotype - translucent to white, glossy, and mucilaginous with embryos of various sizes in a loose mass of translucent cells. Spruce EC arises from subepidermal cell layers of the hypocotyl immediately beneath cotyledons of the explant.

In some species, particularly Norway spruce, both EC and nonembryogenic cultures (NEC) form from a single explant. In such instances, the two types can be isolated and cultured separately. Having both types allowed tests for biochemical differences, and development of markers for predicting embryogenic potential (Wann et al. 1987). In general, EC evolves less ethylene, contains lower amounts of reducing agents, and synthesizes protein at faster rates than NEC. Such markers, however, have limited utility in that conifer EC are easily recognized. Understanding such differences and their causes, however, could yield means for improving initiation.

Initiation from immature spruce embryos is straightforward, and occurs on various basal media, supplemented with auxin and cytokinin. Conditions for initiation from mature embryos are more demanding. Several factors must be altered, and workers have not yet identified the most important.

Initiation frequencies for immature and mature Norway spruce embryos at IPC average 80 and 25 percent, respectively. More than 50 percent of these EC can be maintained and increased with ease. Some have produced large numbers of embryos for over three years. These frequencies are somewhat lower than those of other workers (von Arnold and Hakman 1988a), but the methods are sufficiently reliable to provide adequate numbers of cultures and embryos for research on other process steps. Embryo numbers in spruce cultures range from 200 to 155 per gram of EC.

Obtaining pine EC demanded media much different from those suitable for spruce. Explant developmental stage also proved more critical. Best results were obtained with precotyledonary embryos about 0.5 mm in length. In all pines investigated at IPC, EC originates from the suspensor region of explants. These differences between pine and spruce underscore the difficulty of research on embryogenesis, and the need for understanding underlying processes and mechanisms.

Initiation frequencies were as high as 12 percent in pond pine, but average only 3 to 5 percent for white and loblolly pines. IPC protocols have worked for several pine species, but are not yet applicable to large numbers of genotypes. In loblolly pine, EC was initiated from explants of 80 percent of the parents from which explants were collected, but over 70 percent of the EC formed from explants collected from only three parents. Such findings demonstrate the importance of genotype, and the need for protocols that will yield higher frequencies and work with a wider array of genotypes.

On a more positive note, pine EC have proven easier to maintain and expand than spruce. Over 80 percent of the cultures initiated in the last few

years are still growing well, and producing embryos for tissue culture and biochemical research on embryo development and maturation.

Douglas-fir EC was obtained from immature embryos collected in summer, 1988. Previously, EC formed only on an infrequent and sporadic basis. Even now, frequencies average less than one percent. As in pines, explant developmental stage is critical, and immature embryos are most responsive. The new cultures are growing better than any produced in earlier years, and numerous early stage embryos are evident.

Despite the progress noted above, initiation is still a difficult step in most conifers. Frequencies remain low and vary greatly among species and individual genotypes. Reliable procedures for explants from older trees are lacking. More investigators are now working on embryogenesis, and collaboration among them may lead to faster resolution of these and other problems than would have been possible several years ago.

DEVELOPMENT/MATURATION

Enlargement and development of somatic embryos roughly parallel that of zygotic embryos, and proceed along much the same pathway in the several species studied at IPC. In general, protocols fostering development in one species tend to work for others as well.

Spruce somatic embryos can develop and mature on media lacking growth regulators, but addition of abscisic acid (ABA) greatly improves development (von Arnold and Hakman 1988a). Similar results have been noted at IPC, but adding low levels of indolebutyric acid along with ABA further increases numbers of embryos reaching mature stages. In response to such treatments, spruce

embryos enlarge, form cotyledons, and mature in several or more weeks. ABA additions also stimulate accumulation of lipids, proteins, and other reserves (Hakman and von Arnold 1988 and von Arnold and Hakman 1988b). Our investigators found similar results, but have also quantified the timing and amounts of accumulating reserves. In general, somatic embryos accumulate the same materials as their zygotic counterparts, but more slowly and in substantially smaller amounts. Current work is focused on methods to hasten and further increase accumulation.

On the average, such treatments yield maturation frequencies of three to seven percent in spruce. Such frequencies indicate significant progress, but are far below acceptable levels. Even more regrettably, they appear typical and thus signal that embryo maturation is a severely limiting step. Spruce EC at IPC average roughly 700 embryos per gram, clearly showing the potential multiplying power of somatic embryogenesis. Realizing that potential, however, requires getting more than seven percent to mature. Empirical tissue culture experiments are likely to lower this roadblock, but improved understanding of the physiology and biochemistry of development is also needed.

Maturation of pine somatic embryos has proven even more difficult. ABA treatment provokes responses similar to those in spruce, but development ceases before cotyledons form and neither mature embryos nor seedlings have been obtained at IPC. This seems true for most laboratories, and confirms the need for increased research on this limiting step.

CONVERSION TO SEEDLINGS

Small numbers of conifer seedlings have been recovered by several workers. Best results, however, have been obtained with Norway spruce. For germination, mature embryos are picked individually from EC and transferred to

dilute media minus growth regulators. A short period in darkness is beneficial (von Arnold and Hakman 1988a). Trials at IPC confirm that darkness aids germination, and also indicate that treatments avoiding or minimizing immersion of radicles in agar are also helpful (Becway et al. 1987b). In recent experiments, roughly 40 percent of embryos given these or similar treatments elongated roots and were considered suitable for transfer to potting mix and eventual movement to greenhouse conditions.

Seedlings developing from somatic embryos are quite sensitive to environmental change, and careful control of temperature and humidity are necessary during the transition from culture vessel to greenhouse. Acclimatization can be accomplished by keeping the seedlings in closed containers, slowly reducing the frequency of watering and gradually opening containers to the atmosphere. Fertilizer and fungicide treatments are also recommended, but these and many other requirements are not well understood.

The seedlings first obtained at IPC (Becwar et al. 1987b) recently completed their third growing season. Throughout their lives, they have started and stopped growth in synchrony with zygotic standards. Their phenotypes are generally similar to standards in other respects as well, with the only apparent difference being a tendency for somatic seedlings to have fewer and finer branches. Recently, over 500 new seedlings were transferred to greenhouse conditions. These represent 25 to 60 percent of the germinating embryos used in the experiments. Difficulties during the transition period were numerous, and more reliable procedures for care and handling are needed. These and new seedlings from a wider array of EC will be used for tests of performance, uniformity, and fidelity.

SUMMARY AND CONCLUSIONS

Tree improvement based on classical genetics has increased productivity in many species, and a wealth of breeding material is available for the future. Despite such successes, the process remains time-consuming and costly.

Clear parallels exist between classical and molecular genetics, and a variety of molecular techniques may become effective supplements to the classic tools of selection and breeding. Approaches such as direct gene transfer and somatic hybridization are being developed to add new and different genes. With time, molecular markers and various cell and tissue culture methods may be used to raise selection efficiencies and shorten generation intervals. Just when these and other molecular techniques will be integrated with classical programs is uncertain, but the potential is considerable and prospects are encouraging.

Another element crucial for eventual integration of molecular techniques is an efficient means of asexual propagation. Application of some molecular techniques requires reliable cell and tissue culture methods, and an efficient regeneration system is necessary for moving plant materials selected or genetically altered in culture into breeding or production populations. Somatic embryogenesis is well-suited for such purposes, and could become an effective integrator.

Significant advances have been made in research on somatic embryogenesis, but more work is needed to make the system a useful means of integration.

Effective integration, however, does not require simply "more" research. Needed instead is a balanced mix of research, a mixture that devotes appropriate funding and talent to needs in each of the several areas. Clear goals, a strong

sense of priorities, and stable support are essential lest one area of research outstrip others, with application hindered because not all the right pieces of technology are available at the right time. Care must therefore be taken to preserve the momentum of classical breeding programs, and ensure continued progress across generations. Appropriate and continuing support are also needed to ensure that molecular tools are developed in appropriate order and time, and that an efficient regeneration system is available for use in research on and application of molecular techniques.

Any research organization would benefit from having all the right disciplines working on the species of choice at one location. Since few can afford such luxury, those responsible for planning and direction must provide means and incentives for cooperation and collaboration among disciplines, organizations, and even nations. Ideally, advances in individual areas should proceed in accordance with an overall strategic plan, with specialists in the several fields working together, sharing goals, plant materials, and results.

Orchestrating all this within a government, an industrial firm, or even a single laboratory may prove more difficult than executing the research itself. Given the high costs and long lead times, however, dedicated efforts to manage integration and find solutions to problems, that in reality are common ones, seem far better and more productive than wandering about with a solution in search of a problem.

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