8. Genetics of micropropagated woody plants

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1. Introduction

To perform genetic studies with any organism, it is imperative that a source of variation be available. In the past variation has come from wide crosses, germplasm collection and utilization, mutagens, and sports. With the advent of tissue culture protocols, variation has been ubiquitious. The variability associated with tissue culture has provided a pool of variation upon which selection pressure has been imposed to isolate unique forms of clones. this variation, known as somaclonal variation, has become important for plant improvement, but the genetic basis of this variation remains largely unknown. In this chapter we will explore different types of variation and then discuss relevant investigations that have been used to elucidate the genetics of the variation.

2. Tissue culture as a source of variation

The amount of variation arising *in vitro* depends on the clone, age of the culture, use of mutagenic agents, and selection pressure applied to single cell clones for stress conditions such as salt level, microorganism or their byproducts, and specific metabolites. It is even possible to select plants for cold tolerance *in vitro* [29]. The exploitation of such natural and induced variation seems especially applicable to long established woody plant cultivars, such as 'Bartlett' pear and 'Delicious' apple (introduced in 1770 and 1893, respectively), which could be expected to have accumulated large numbers of mutant cells that may have stabilized into cell mixtures (chimeras) of various complexity [229].

2.1. *Natural variation and 'sports' as sources of variation*

The use of asexual methods for clonal improvement of woody plants is not new. In fact, many trees and treelike monocots, which are grown extensively

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for their fruits and oils, are propagated vegetatively [95]. The improvement of these horticultural and plantation crops, such as oil palm, coconut, rubber, peach, apple, walnut, relies heavily on cloning of unique genotypes that have arisen through bud mutations called 'sports' [229]. For instance, over 150 bud sports of 'Delicious' apple have been named as unique cultivars [75]. The genetic basis of certain sports have been studied and some traits are transmitted through the sexual cycle. For instance, the 'thornless' condition of 'Thornless Evergreen' and 'Thornless Loganberry' blackberry obtained *in vitro* [97,160,161] is due to dominant genes which have named '*Ste*' and '*Sfl*', respectively [96,208]. The topic has been reviewed by McPheeters *et al.* [161a].

Some mutations have proven to be so unique that they are of little value to the geneticist for inheritance studies. For instance, it is reported that the Seedless Navel orange arose as a bud sport on a seeded [246] orange tree in Brazil. The seedless character has proven to be extremely important economically, but its sterility limit its importance as a female parent. Recently hybrids of Navel and mandarin orange were produced by somatic hybridization [134]. Perhaps this hybrid plant can be used to elucidate the genetics of parthenocarpy.

There are many ways that variability can be obtained *in vitro* [85]. We discuss several types and sources of variation and then to briefly review literature related to the topic. Finally, we present examples to illustrate how the topic either has been used for genetic analysis or how such studies could begin.

2.2. *Types of tissue culture variation*

There are many types of variation that one can encounter *in vitro* [125a]. These include changes in growth habit of callus, habituation [162,163], variability in ability to regenerate, biochemical requirements and sensitivity (see Rains *et al.* [189] for a review of salt tolerance), disease resistance [209], changes in chromosome constitution [39,146,175] and changes manifested in whole plants. The subject has been reviewed by several authors [26,131,141,143, 153,163,227,230]. Entire books on the subject have been published [53,100,253].

2.3. *Sources of variation*

Cellular variation can result from either genetic mutation, epigenetic change, or a combination of both. Meins [163] suggests that genetic mutations involve random alterations in genetic constitution such as point mutations, deletions, duplications, and rearrangements of the genetic material. Epigenetic changes primarily involve time or tissue-specific 'selective gene expression rather than sorting out of genetic determinants' [163].

Meins [163] suggests that genetic mutations can be differentiated from epigenetic changes through several simple steps: (1) epigenetic changes are

directed, i.e. it occurs regularly in response to specific inducers. Under these conditions, the rate of epigenetic change is high, greater than 10^{-3} per cell generation. In contrast, well-characterized gametic mutations in plants occur at rates of less than 10^{-5}; (2) the variant phenotype may be stable but it is potentially reversible and the reversion occurs at high rates; (3) The range of phenotypes generated are limited by the genetic potential of the cell; and (4) epigenetic changes are not transmitted meiotically. For instance, Dix and Street [48] reported a callus line of tobacco that was resistant to chilling. When the callus was subcultured, some clones retained the chilling resistance through two subclones; most showed no enhanced resistance. This reversion to the wild type suggests that the chilling effect was epigenetic, not genetic [163].

When variant cells remain totipotent, mutations can be distinguished from epigenetic changes by regenerating plants from variant clones and assaying tissues from the *R* generation and its selfed offspring. For a good test of heritability, it may be necessary to continue the selfing into at least the *R*2 generation [163].

Stable epigenetic changes can be used to obtain unique characteristics such as variegation, modified growth habit, lack of thorns, etc. Since this type of variation is not sexually based, it is useful only if it can be maintained by asexual propagation systems such as cuttings, layerage, or tissue culture. It cannot be passed sexually to another cultivar. Epigenetic changes are not permanent because they involve a network of gene(s) that are 'turned on' at some specific stage of development and turned 'off' later, however a plant may remain in the activated state indefinitely. The process of meiosis resets the gene network to a 'ground state'. On the other hand, changes with a genetic basis, can be maintained asexually or they can be exploited via the sexual system to improve many cultivars through breeding systems.

Where does variation come from? Gould [85] has reviewed this topic. In some cases the variation may be preexisting. For instance, when a complex explant is placed *in vitro*, it is unlikely that all cells will proliferate equally. For this reason, selection can begin very quickly *in vitro*. This is especially noticeable in new cultures where cultures often change in color, friability, and growth rate within the first few subcultures.

With time one cell line can be preferentially selected over another, resulting in the loss of the original genotype. It is also possible that a presumed pure culture can consist of more than one cell type. For instance, cytokinin habituated cells of tobacco can produce sufficient growth factors to support the proliferation of non-habituated cells in the same tissue [241].

The source of variability *in vitro* also can be traced to changes which are associated with passage through the tissue culture environment [66,67,68,168, 214]. Morrison *et al.* [168] suggest that 'much of this variation may be related to substances in the culture media that act as mutagens or maintain a degree of cell division for the plant cell's repair mechanisms cannot keep pace'. For instance, Evans and Bravo [64] report that ornamental tobacco plants re-

generated from media with 6-benzylaminopurine exhibited more variation than cells grown without this cytokinin.

In other cases, the variation can be induced by mutagenic agents or by the tissue culture process itself [19,64]. These possibilities are discussed later in this chapter.

Although there have been numerous reports of 'mutation' in culture, few have been verified by breeding experiments (for a sample of the literature on the subject see Refs. 4 and 100).

To study the genetics of micropropagated plants, most reserachers follow the recommendation of Cheleff [26] who suggested that plants derived from cell and tissue culture be called the '*R*' generation. Progeny obtained by self-fertilization of the regenerated plants can then be designated as the '*R*1' or '*R*2' generation etc. for each successive sexual generation. This designation will be used in this chapter.

2.4. *Accessing variation via* in vitro *techniques*

To access natural or induced variation, it is necessary that a regeneration system be developed whereby whole plants can arise adventitiously from single cells or small groups of cells. Furthermore, the use of tissue culture or genetic engineering systems for genetically improving woody plant species requires a reliable regeneration system. Unfortunately, many woody plant species are recalcitrant.

Although adventitious organogenesis has been achieved in several woody species [7,114,195,231], most regenerants have been from juvenile tissues of seedling origin. Direct improvement of woody plant cultivars requires the control of regeneration from mature tissues. Promising results have been obtained from apple [61,70,118,122,256], *Prunus* [50,117] and pear [31,173a]. The subject of somatic embryogenesis in woody plants has been reviewed by Tulecke [249]. The introduction of thidiazuron (a cytokinin-like substance) has made many previously recalcitrant woody crops yield to the tissue culturist [31,70,129]. As the ability to regenerate becomes more common among the woody plants, the importance of tissue culture biotechnologies for woody plant improvement will increase.

The sources of variation which we have chosen to discuss in further detail below are (a) somaclonal variation, (b) genetic manipulation via genetic engineering, (c) embryo culture or rescue, (d) *in vitro* pollination, (e) haploidy, (f) chimeral segregation, and (g) protoplast fusion.

3. Somaclonal variation

Somaclonal variation is defined as genetic variation observed among progeny of plants regenerated from somatic cells cultured *in vitro*. Somaclonal variation

is now known to be widespread among tissue culture-derived plants [25,26, 141,153,161,163,173c,174,227,230], but its genetic basis largely remains unknown in both woody and herbaceous species. The genetic basis of the phenomenon is seldom discussed, particularly in early reports [168]. Although the cause of somaclonal variation is often not known, this variation has proved useful in classical breeding programs [67,68,96,97,98].

Somaclonal variation has been observed among woody plant regenerants. The subject has been reviewed by Ahuja [3a]. Lester and Berbee [150] observed variation in height, number of branches, leaf traits, and chromosome number among callus-derived plants of *Populus nigra* and *P.* × *euramericana*. Callus cultures and regenerated shoots of *Citrus* and grape [228a] have shown tolerance to sodium chloride [15,234]. In a field test, micropropagated loblolly pine plants have shown a higher mortality, less shoot growth and more curved stems then seedlings [143a]. Micropropagated aspen (*Populus tremula*) cultures showed differences in root morphology [2a].

Many traits have been indentified from somaclonal variation studies [168], but few of these have been analyzed genetically. The lack of genetic analyses is not due to lack of interest by the researcher. Genetic analyses can be slow and time consuming, particularly for woody tree species. Although the genetic basis of somaclonal variation has not been subjected to thorough analysis for woody plants, its nature has been explored in herbaceous crops.

Herbaceous species have been used to elucidate the genetic nature of somaclonal variation. Some of this research will be discussed as a model for those interested in woody plants. The best work to date has been done with tomato (*Lycopersicon esculentum*), an herbaceous perennial that is self pollinated, true breeding and is usually seed propagated (see refs. [62,66,69,226] for more detail).

Evans and Sharp [66] began their studies by establishing cultures of tomato from leaves of germinated seedlings. Among the regenerants were detected chromosomal variants, single cell changes, and cytoplasmic genetic variants [168].

Some of the somaclones proved to be mutants of the parental cultivar. In one experiment 13 single gene mutations were observed among 230 regenerated plants. The traits ranged from changes in fruit color to jointless pedicels [66]. Genetic analyses were performed by either evaluating selfed *R*2's that had been selected in the *R*1 cycle or by crossing with known mutants. For instance, in the case of fruit color several progeny had yellow instead of red fruit. The yellow fruited variant was crossed to known yellow-fruited mutants to identify the gene's location in the tomato genome. In this manner, it was demonstrated that the somaclonal yellow mutation was on chromosome 3. Similar strategy was used to identify orange fruit color on Chromosome 10 and jointless pedicels and resistance to *Fusarium oxysporum* reside at opposite ends of Chromosome 11 [68,168]. Morrison *et al.* [168] also reported that some of their variability resulted from mitotic crossing over. They demonstrated this fact by using a tomato line heterozygous at four marked loci on

chromosome 6. Among 61 regenerants, 19 exhibited recombination for one or more of the markers. Lee and Phillips [146] have published an entire review on the chromosomal basis of somaclonal variation.

Another source of variation may have been changes in organelle DNA. For instance, Kemble and Shepard [128] detected changes in mitochondrial DNA but not chloroplast DNA among potato plants regenerated from protoplasts. The importance of such variation is discussed elsewhere in this chapter.

An additional form of variation among the tomato regenerants involved changes in a regulatory gene which caused virescence in the leaves of tangerine-colored fruit. This character proved to be a single recessive gene which probably regulated the amount of chlorophyll in young leaves but not older leaves [168]. One somaclone had resistance to *Fusarium* race 2, due to a single dominant gene mutation [63.].

In some cases, genetic analysis may be impossible. Shepard and his colleagues, for instance, gained recognition for their work with protoclones of the herbaceous perennial 'Russet Burbank' potato (*Solanum tuberosum*) and its extreme variability. Variant somaclones included such diverse characters as stem length, number of blooms, weight of #1 tubers, tuber specific gravity, number of tubers, etc. [215,221,222)]. The variability was field tested and some types were stable [193]. These clones were perfect candidates for genetic analysis, but, unfortunately, the 'Russet Burbank' cultivar has sterility problems. Although classic genetic analyses were not possible, Shepard's group did examine the chromosomal status of their clones and concluded that much of the variability could be attributed to chromosomal rearrangemenets [128]. An interesting conclusion is that the somaclonal variation system is suited well for improvement of plants which are fully or partially sterile but maintained as a clone.

Some types of sterility can be analyzed sexually. For instance, some types of seedless fruits actually set seed, but the embryo aborts prior to maturity. However, it lives long enough to produce sufficient hormones to cause fruit expansion. Embryo rescue systems have been used to obtain sexually-derived seedlings of grape [83] and *Prunus* [192]. Modified versions of this technique could be used to rescue embryos of sterile somaclones. Bajaj and Gill [9] have carried the system one step further by returning embryo rescued *Gossypium* hybrid seedlings to tissue culture to induce somaclonal variation in the hybrid.

Somaclonal variation among forest trees has been briefly reviewed by Ho and Zsuffa [110]. Lester and Berbee [150] reported variation in height, branching and leaf traits of a *Populus* hybrid; variability was associated with unstable chromosome counts. Cheng and Smeltzer [30] found loblolly pine regenerants with changes in needle morphology and number.

3.1. *Selection and screening of disease resistant plants*

Woody plant cultivars are constantly attacked by various insects and fungal

and bacterial diseases. Control requires pesticide applications throughout the growing season. The use of tissue culture and other techniques of biotechnology may be useful to either isolate genotypes with reduced susceptibility to disease or transfer disease resistance genes without altering other characters such as fruit quality. The strategies of selecting and evaluating for disease resistance *in vitro* have been discussed by Miller and Maxwell [165] and Larkin and Scowcroft [142] who used their sugar cane cultures to obtain somaclonal variation for *Helminthosporium* resistance. Shepard [221] reported potato protoclones with increased resistance to early and late blight. Evans [63] reports a tomato somaclone with a dominant gene that gives it resistance to *Fusarium* race 2. The subject has been reviewed by Sacristan [209].

Larkin and Scowcroft [142] reported on the isolation of *Saccharum* somaclones with increased resistance to *Helminthosporium sacchari*. Sexual analysis of the trait was impossible due to very high polyploidy and sterility problems. Therefore, the authors chose to assay the stability of the character through several vegetative generations (of 85 somaclones, 73% were stable in their reaction to toxin; 8% reverted to some degree; and 19% segregated). Six toxin-tolerant lines were carried through a second generation of tissue culture and maintained as callus for 3 to 6 months prior to regeneration. Three of the progeny segregated; three were stable. Among 60 somaclones derived from the second cycle of tissue culture, 40% had tolerance similar to the parent clone, 22% were more tolerant and 38% were more susceptible. This suggests that through repeated cycles of tissue culture, it may be possible to continue to select desirable characters while retaining previously selected characteristics.

Somaclonal variation has been used to obtain a putative *Septoria* leafspot-canker tolerant hybrid poplar. These are being greenhouse and field tested to assess the true situation [60]. Barlass *et al.* [12b] have tried to screen for resistance to Downy Mildew in grapes by establishing dual cultures of grape and pathogen. Joung *et al.* [123] has screened apple cultures for resistance to cedar-apple rust *in vitro*.

3.2. *Selection and screening of herbicide- and salt-resistance*

3.2.1. *Herbicide resistance*

The control of weeds is a worldwide problem that has proved ideal for *in vitro* manipulations. As long as a researcher has access to a regeneration system, it is possible to grow cells or organs in contact with a specific herbicide (or toxin, metabolite, salt, etc.) and select cell lines that have improved tolerance or, perhaps, resistance to the compound. The possibility has stirred much interest. The status of such research has been reviewed [27,37,113,257].

The best known example of herbicide resistance is that reported by [219] who were able to select a line of tomato with resistance to glyphosphate *in*

vitro. The nature of the tolerance was later shown to due to amplified gene copies of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, the enzyme that is inhibited by glyphosphate. Transgenic plants containing the EPSP synthase gene produced more of the enzyme and are relatively resistant to glyphosate. In another case, a bacterial gene which confers resistance to glyphosphate was isolated from *Salmonella* [36]. Glyphosate tolerane has been transferred to many species [257] such as tomato and potato, via the Ti plasmid. Glyphosate-tolerant *Populus* plants have been reported by Fillatti *et al.* [72]. The herbicide tolerance factor can be expressed in the whole plant, but not always at agriculturally significant levels [37,257].

3.2.2. *Salt tolerance*

Salt-tolerant cell lines of many herbaceous species have been selected. The subject has been reviewed by Rains *et al.* [189]. Salt tolerant *Citrus* [15,135,234] and grape [228a] cell lines have been selected *in vitro*. Labrun *et al.* [144] showed that cotyledon and hypocotyl tissues of grape embryos were more tolerant of NaCl than root tissue. Salt tolerant plantlets were derived from *Citrus* callus selected on relatively high [15] and low [234] levels of NaCl. The plantlets selected on high salt were so abnormal (without internodes) than they could not be cloned for further assessment. Plantlets selected on lower NaCl levels were more normal in appearance and expressed some salt tolerance.

4. Genetic manipulation via genetic engineering

Virtually all plant genetic engineering involves tissue culture or micropropagation at some stage, hence a brief discussion of genetic engineering is relevant to a general discussion of genetics and micropropagation. The subject has recently been reviewed for woody plants by Ahuja [4]. The relative difficulty of manipulating woody species by classical genetic systems, coupled with their ability to be vegetatively propagated on a commercial scale, makes the application of genetic engineering to woody plants attractive. In the following section, we first address the status of genetic engineering in herbaceous plants; we then summarize progress in woody angiosperms and gymnosperms.

4.1. *Candidate genes*

Genetic engineering is currently limited to characteristics that are primarily controlled by the expression of single genes (see [84] and [78] for reviews). However, most agronimically important traits are controlled by multiple genes, and the role of specific genes in basic growth processes is largely unknown.

As a consequence, a common strategy for genetic engineering in plants is to introduce genes that confer novel functions. For example, resistance to the herbicide bromoxynil is conferred by a bacterial gene (*bxn*, isolated from *Klebsiella ozaenae*) encoding a nitrilase that detoxifies bromoxynil by converting it to 3,5-dibromo-4-hydroxybenzoic acid [236]. A similar strategy has been used to engineer resistance to the herbicide phosphophinothricin [42,244]. Genes encoding the insecticidal toxin from the bacterium *Bacillus thuringsiensis* have been introduced into several plants where they confer resistance to insect herbivory [13,74,251]. Finally, plants containing genes that encode viral coat protetins from tobacco mosaic virus appear resistant to viral infection [14,45,198].

While genes that impart a novel function or product often originate from prokaryotes, they may also come from plants. Genes encoding proteinase inhibitors have been isolated from potato [210,211] and cowpea [107]. The cowpea gene confers resistance to herbivorous insect pests in transgenic plants [107]. Resistance to glyphosate in plants may result from introduction of a gene from *Salmonella* [36], or from a plant gene. A gene encoding 5-enol-pyruvylshikimate-3-phosphate (EPSP) synthase (the enzyme inhibited by glyphosate) has been isolated from tomato (discussed earlier). Transgenic plants containing the EPSP synthase gene produced more of the enzyme and are relatively resistant to glyphosate [219]. In this instance, herbicide resistance is mediated by modifying an endogenous function, i.e. increasing the abundance of a specific protein.

It is more difficult to reduce or repress expression of endogenous genes, but methods are now becoming available. One approach involves introduction of genes encoding antisense RNA, RNA transcripts that are complementary to those of an endogenous gene. Antisense RNA is believed to interfere with processing of normal RNA such that normal gene expression is inhibited [55]. In plants, antisense RNA appears effective in reducing the expression of introduced [44] as well as endogenous [206,220] genes. More recently, RNA enzymer (ribozymes) with endoribonuclease activity have been engineered to have high specificity for target sequences [103]. Antisense RNA and ribozymes both offer the possibility of introducing genes causing dominant loss-of-function phenotypes.

A final technology offers unique opportunities to identify and isolate genes with important biological functions [213]. Gene-tagging involves the insertion of a foreign segment of DNA into a gene which in turn disrupts its normal expression (see Shepherd [224] for review). If the inserted DNA segment has previously been cloned, and if the phenotype is noticeably altered by the disruption, the altered gene can be indentified using the inserted segment as a tag. Once cloned, the altered gene can be used to isolate a copy of a normal gene. Because of their ability to move in the genome, transposable elements are ideal for gene tagging. While transposable elements have been cloned from only a few plants, some transposable elements (e.g. *Ac* from maize [10,11] retain their ability to move after transfer into plants belonging to a different

species from which they were isolated. Therefore, gene tagging using transposable elements may be broadly applicable in plants, even in species for which transposable elements have yet to be identified and cloned.

In terms of promising candidates for genes to manipulate, woody plants suffer the same difficulties of their herbaceous counterparts, but the problem is exacerbated by their typically long generation intervals. Relatively few single-gene traits have been characterized by traditional genetic analyses in woody plants, and even fewer have potential for improvement of commercially important traits [145,216], but see Thompson [245], for an interesting candidate). Application of genetic engineering to woody plants will endoubtedly benefit from experience with herbaceous plants. However, for the many growth processes that are unique to woody plants (e.g. maturation, leaf senescence, nutrient translocation, perennial habit, wood formation, etc.), research on herbaceous species is not sufficient. Efforts must be made to direct research towards the unique characteristics of woody plants.

The best example to-date of the application of genetic engineering to a woody species is the introduction of herbicide resistance into *Populus* [72], although other systems are currently being explored, e.g. walnut [157] and apple [116].

4.2. *Regulating transferred genes*

Once a specific gene has been identified and transferred, optimal utility can be realized only if its expression is properly regulated. Among the many factors regulating gene expression, cis-acting DNA sequences such as those associated with enhancers, promotors, or introns are all being investigated [24,71,126,154, 177,178,254]. Similarly, proteins that interact with DNA directly are being characterized [81,87,88,119,120]

While mechanism regulating gene expression are incompletely understood, empirical studies have yielded useful generalizations. Genes that are highly regulated (either developmentally or in response to environmental stimuli) normally are also highly regulated following their transfer to other plants of the same species or closely related species [184]. It is not clear how similar target and donor species must be for a transferred gene to function properly. However, some regulatory processes appear more sensitive to differences in genetic relatedness than others [57,81,112,127,151,255]. Furthermore, most regulatory functions are intrinsic to the specific sequences (i.e. regulatory sequences) that regulate expression of the protein or RNA (i.e. structural) coding sequences with which they are associated. Hence, regulatory sequences from a light-inducible gene such as the small subunit of RUBP-carboxylase can be used to control expression of structural genes for which a similar pattern of expression is desired [35,247]. These generalizations are encouraging for future applications because it will be possible to engineer DNA molecules that contain coding sequences for a desired attribute expressed in a desired fashion.

4.3. *Gene transfer methods*

Use of an effective vehicle to move a desired gene into plants is a crucial and often limiting step in genetic engineering. To date most transgenic plants have resulted from use of *Agrobacterium tumefaciens* [80,105]. A related species, *A. rhizogenes*, has also been used). These organisms harbor a tumor-inducing (Ti) plasmid, and as part of their pathogenic life cycle, transfer a portion (transfer-DNA or T-DNA) to the chromosomes of their plant host. Isolated T-DNA can be modified *in vitro* to include genes of interest, and reintroduced into bacterial cells. As these cells subsequently infect a plant, host cells receive the modified T-DNA that includes the foreign gene. Problems related to the use of *Agrobacterium* result from host range limitations, as well as in regenerating plants from transformed cells.

Several approaches have been devised to circumvent the biological limitations to *Agrobacterium*. Perhaps the most popular has been to introduce DNA directly into protoplasts (see Shillito and Saul [225] for review of general methods). While assays of transient gene expression can be done for any plant for which viable protoplasts can be isolated, transfer of foreign genes into plants is limited to species for which plants can be regenerated from protoplasts. Because of its broad success in animals, microinjection into plant cells appeareed promising. Unfortunately, there are only a few reports of successful gene transfers [38,199,200,201], and these were done using protoplasts. Recently, bombardment of intact cells with microprojectiles has stirred considerable interest [20,32,132,133,158]. The method requires relatively little preparation of the recipient specimen, it appears amenable to a variety of organs and species, and can be used to transfer genes into different organelles. In addition to transformation of recalcitrant species such as soybean [32,158], microprojectiles have now been used to transfer genes into Douglas fir [82] and loblolly pine (A. Weisberg, North Carolina State University, pers. comm.).

Transferring genes and ensuring their regulation are intimately intertwined, so results and progress in these areas for woody plants are discussed together. Experience using *Agrobacterium* in woody plants is limited, but growing. In angiosperms, transformation has been reported for *Populus* [73,176,187], English walnut [40,157], olive [239], peach [99], citrus [166], apple [8,116], grape [12a,33,138], and willow [252]. Transformed plants have also been regenerated [73,116,157,239]. *Agrobacterium*-mediated transfer of genes into other woody angiosperms is being actively pursued.

The host range of *Agrobacterium* had been reported to exclude most gymnosperms [43]. A survey of additional *Agrobacterium* strains has identified several capable of infecting recalcitrant conifers such as pine [217,238], and gene transfer has been verified (Loopstra *et al.* in prep.). Agrobacterium-mediated transformation of spruce has been reported [56,111], and studies involving a number of other conifers are under way.

These studies demonstrate that routine procedures for herbaceous plants can also be applied (often with somewhat greater difficulty) to woody plants.

Similarly, DNA constructs (i.e. a regulatory sequence coupled with an appropriate structural sequence) that work well in herbaceous plants often work well after transfer to cells of woody plants, including conifers [16,82, 92,159,218]. These results are encouraging, but caution is warranted because they are limited to gene constructs under the control of relatively strong promoters that are constitutively expressed in herbaceous plants. Highly regulated genes have not yet been studied.

5. Embryo culture and rescue

Embryo culture techniques have direct value to the plant breeder who is trying to make crosses among plants with fertilization barriers of various sorts. It is also important as a support system to facilitate genetic studies with families, genera, species, and cultivars which are normally not sexually compatible [203]. Embryo culture and rescue systems can also aid in germination of recalcitrant seeds [91]. Barriers such as ploidy differences, chromosome alterations, and genetic incompatibility could lead to embryo abortion. The situation is often seen in hybridization studies of interspecific or intergeneric type [6,110]. Embryo culture can also be used directly for biochemical studies such as that reported for cocoa butter accumulation in *Theobroma cacoa* [136]. Dunwell [51] has summarized the literature concerning the use of embryo culture to rescue interspecific hybrids.

Embryo culture can be accomplished by either *in ovulo* (where the embryo and its supporting tissues are grown *in vitro* or embryo rescue systems. About 50 interspecific and intergeneric hybrids have been obtained through *in ovulo* embryo culture systems [110]. Most of the earliest work was done with herbaceous species; there are recent reports of success with woody perennials. *In ovulo* techniques have had the most value in the breeding of seedless grapes. For instance Emershad and Ramming [58] reported the technique which was further developed by Spiegel-Roy *et al.* [235], Gray *et al.* [86] and later by Emershad *et al.* [59]. In this manner genotypes which could not have been used as female parents, due to seedlessness, can be used for genetic studies.

The method has also been used to obtain interspecific hybrids between incompatible species as *Carica papaya* and *C. cauliflora* [260]. Ramming [192] has used the technique to culture small embryos from early maturing *Prunus* genotypes which normally abort prior to fruit ripening. Kouider *et al.* [137] and Savka *et al.* [212] have used the system to rescue embryos of *Populus deltoides*.

Limited success with pine proembryos [139,242] and Norway spruce [140] has been reported. Proembryo degradation is thought to be due to genic and cytoplasmic incompatibility. Special techniques such as 'nurse cultures' have been adopted to overcome the difficulties encountered in culturing proembryos [242].

Nucellar embryony is a phenomenon commonly observed in species such

as *Citrus* and *Mangifera* [194]. Nucellar embryos of these species can develop into virus-free juvenile progeny. The nucellar system has been expanded to grape [170] and monoembryonic *Citrus* [194].

6. *In vitro* pollination and fertilization

In vitro pollination and fertilization are most useful when the two parents are incompatible due to problems associated with reproductive organs or flower abscission prior to seed maturity [110]. The first successful use of this system was demonstrated for poppy (*Papaver*) [125]. Although the *in vitro* culture and pollination of floral parts may have value, we are not aware of any reports where these systems have been successfully for woody plants. The system has been used succesfully for a number of herbaceous species including maize [106] and cucumber [169].

6.1. *Haploids in tissue culture*

Haploid plants are of importance for plant breeding for several reasons: (1) homozygous plants can be obtained in one generation, even from self-sterile plants; (2) large numbers of haploid progeny can be obtained. Because dominance cannot mask expression of desirable alleles, the time required to select desirable genotypes can be reduced [167]; (3) haploid plants are better plants for inducing and detecting mutations than diploid plants [47] (the name given to this type of variation is gametoclonal [69]); and (4) for those plants that develop from single pollen grains, the problem of chimera induction via mutagenesis is reduced. (The lack of chimera formation has also been reported with protoplasts [18].)

Substantial progress has been made in the production of haploids during the last decade. The procedures have been used widely to produce disease-resistant and high yielding varieties [172,258]. Although haploid plants have been reported in many angiosperm species, there have been only a few reports on tree crops [108].

Haploids are usually obtained by anther culture. However, plants derived from anthers can be either maternal or pollen-derived. Distinguishing the two types is possible by counting chromosomes, but haploid cells frequently double spontaneously. Without markers the doubled haploids and the maternal-derived shoots are difficult to distinguish. A more reliable method to obtain haploid lines is by pollen culture. The status of this field has been reviewed [17,18,104,152].

Production of haploid plants of woody species appears to be much more difficult than for herbaceous plants [188]. Radojevic and Kovar [188] noted only 25 species of woody plants had been investigated, with successful results from only two of these. Typical results are those reported for grape (*Vitis sp.*)

where only three of 26 clones obtained from anthers survived [89]; none was haploid. Chang-jie and Peifen [28] reported the obtention of haploid grape plants with many aneuploid cells, but no geneticc studies were reported on these plants. Rajasekaran and Mullins [190,191] produced plants from grape anthers but all of the plants were diploid.

Production of haploid plants of several *Populus* species and hybrids have been reported by Hyun *et al.* [115] and Uddin *et al.* [250]. These workers described procedures for plant production from anthers cultures of *Populus deltoides*. They noted differences in leaf morphology and growth of established plants from the same clone. Chromosome counts of regenerates showed more variability than reported by Ho and Raj [109].

6.2. *Gymnosperm haploids*

To date only a few gymnosperm species have yielded plantlets through androgenesis and gynogenesis [18]. One would expect that it will be easier to obtain vigorous haploid plantlets from megagametophyte cultures of tree species such as *Pinus resinosa* than those of more heterozygous species [77a,227a]. However, progress in this area has been slow due to the poor regenerative capacity of cells and tissues of gymnosperms [173]. Perhaps this is to be expected given the extent of allelic heterozygosity and large genetic loads in conifers [78a,233a].

6.3. *Genetic studies with haploid-derived plantlets*

The first homozygous clone of apple was obtained *in vitro* by Milewska-Pawliczuk and Kunicki [164] from microspores. Later Lespinasse *et al.* [148,149] and Zhang *et al.* [261] obtained haploid plants from anthers of apple using a unique genetic based anthocyanin marker system. Duron and Lespinasse [52] have reported that one haploid plant was grown in a phytotron until it was old enough to flower (2 years). The pollen has been used for breeding.

6.4. *Problems of working with haploids*

The problems associated with haploid culture have been discussed by Bonga *et al.* [17,18]. The major limitations to haploid breeding are (1) lack of techniques to obtain large numbers of verifiable haploids since tissue culture is known to drive cell populations rapidly to polyploidy and aneuploidy [171,248]; (2) most trees are highly heterozygous and contain large numbers of lethal and semi-lethal recessive genes. Therefore homozygous plants could be of low vigor and may not grow to reach sexual maturity; (3) the importance

of haploids for tree crop improvement has yet to be demonstrated; and (4) diploidization is difficult in some species.

7. Chimeras in tissue culture

Many woody plants are known to be chimeral in nature. A chimera consists of cell sectors or tissues which differ in genetic constitution but have developed from a meristem containing layers or sectors of mutated tissue [102]. Dermen [46] has described many chimeras. (See Whitham *et al.* [259] for an interesting discussion of evolutionary value of chimera formation and maintenance in woody plants.)

The arrangement of the genetically different tissue within the plant meristem affects chimeral stability. The most stable chimera is the periclinal chimera, however, changes sometimes occur by cell displacement and replacement [237]. It also has been noted that the rate of cell division in the apical meristem can be altered by environmental factors [182], this can affect the chimeral status [12]. Pillai [180] and Reeve [197] reported that stability in the tunica layer of woody plant apices can be a seasonal occurrence.

The most common cause of chimeral dissociation is adventitious shoot production [156]. Adventitious shoots can be produced either *in vivo* and *in vitro*. Adventitious shoots usually arise from a single cell or a few cells that could be derived from specific tissue [21,22]. If adventitious shoots can be traced to a single cell, then any system that yields adventitious shoots will result in chimeral segregation.

Dermen [46], Stewart and Dermen [237], and Dayton [40a,41] have used a technique known as disbudding (removal of all preformed buds on shoots) to stimulate formation of adventitious shoots and separate the components of the cytochimeral apple trees. Other methods to separate chimeras include the use of irradiation [121].

Chimeras, when separated into their component parts, have been used to produce new strains of standard fruit cultivars [96,97,98,160]. However, the lack of a system to separate fruit tree chimeras into pure types has limited their use for clonal improvement. The development of procedures whereby shoots could be formed adventitiously from single cells could speed the development of new types of standard cultivars.

Tissue culture techniques, may offer more control over phenotype selection and propagation than disbudding or other methods. Various explants can be stimulated to produce shoots adventitiously rather than by a pre-formed bud system, thus a chimeral plant, subjected to this system, will segragate into its component genotypes with a chance of new chimeral rearrangements.

Dommergues and Gillot [49] were able to separate the histogenic layers of an unstable *Dianthus* chimera to form a new type that was stable enough to be more commercially tolerable. Opatry and Landa [175] utilized a chlorophyll chimera of tobacco to regenerate phenotypically pure plants from explants

taken from variegated leaves. Kameya [124] was able to separate the green and white portions of variegated leaves. Skirvin and Janick [232a,233] produced nonchimeral *Pelargonium* plants through *in vitro* culture of stem tissue. One of these tissue culture-derived plants was introduced as a named cultivar, 'Velvet Rose' [232b]. Johnson [121] was able to separate a periclinal carnation chimera by using a macerated shoot tip culture technique. Bush *et al.* [23] found *Chrysanthemum morifolium* 'Indianapolis' (a periclinal chimera) was unstable in both shoot tip and callus cultures. Another periclinal chimera of 'Indianapolis' chrysanthemum cv 'CF # 2 In. Bronze' was not stable in shoot tip culture with both multiple stem system (shoot proliferation) or callus system [54].

8. Genetic studies

McPheeters and Skirvin [160,161] have used tissue culture to produce over 1000 plants from shoot tip of 'Thornless Evergreenn' (TE) blackberry. TE is a periclinal chimera in which the histogenic layers of the apical meristem differ in genotype. The outer layer (LI), which produces the epidermis has mutated to a thornless phenotype, while the internal layers (LII and LIII) retain the wild thorny genotype. Parental TE produces thorny rootsuckers; shoots derived from the LI of the TE were nonchimeral and produced pure thornless rootsuckers. About half of the tissue culture-derived propagules showed a normal vining growth habit, the remainder were dwarfed due to the shortened internodes. Adventitious shoots (subepidermal origin) were produced from isolated root segments of the regenerants: full sized plants produced thorny suckers while dwarf plants produced thornless suckers. This indicated that the dwarf plants had originated from the thornless maternal epidermis. In addition both dwarf plants and their pure thornless root suckers possessed a unique bent petiole morphology that could be used as a marker for the pure thornless condition. Hall *et al.* [97] have determined that the bent petiole character is linked to a dominant thornless gene (*Ste*) that is transmitted sexually.

A similar dominant thornless gene has been identified from 'Thornless Loganberry' [96] and named *Sf1* [208]. The pure thornless type was released to the public as 'Lincoln Logan' [98].

The field performance and phenotypic stability of thornless blackberries propagated by tissue culture and tip layer or stem cuttings were compared by Swartz *et al.* [240] they found that tissue cultured plants were phenotypically like tip layered plants and they performed similarly in the field. However, some differences in leaf size and shape were observed.

The genetic stability of micropropagated 'Loganberry' plants was followed for three years by Rosati *et al.* [207] by selfing and evaluating of the *R*1 progeny. 'Thornless Logan' is a periclinal chimera in which the LI involves the thornless character and both LII and LIII are thorny. The LII carries

the thorny character as homozygous dominant. They found that the micropropagation did not affect the stability of the character in LI with 1.5% of the plants with a thorny sector were found in 1982, 1.6% in 1983 and 4.6% in 1984. The stability of the thorny character in LII was assessed by selfing one flower per plant. They found that all except 6 progenies had thorny seedlings. Two progenies of the 6 had thornless seedlings. For LIII all the suckers observed in 358 micropropagated plants were completely thorny.

Adventitious shoot propagation of 'Meunier' grape vine (a periclinal chimera) resulted in three different plant types, one group of plants resembling the 'Meunier' another resembling the 'Pinot Noir' and the other group bearing tomentose leaves with hairless sectors. This micropropagation system allowed the separation of the chimeral 'Meunier' into its genotypes [228].

Decourtye [43a] and Chevreau *et al.* [31] have reported that 'Louise Bonne Variegated' and 'Comice Variegated' pear cultivars have chlorophyll deficiences that are under cytoplasmic control. Decourtye [43a] found that the 'Max Red Bartlett' cultivar has a single dominant gene coding for anthocyanin, the same gene found in the cultivar 'Royal Red Hardy'.

Abu-Qaoud *et al.* [1] were able to separate two chimeral pears into their component genotypes using adventitious regeneration system. With this system 'Louise Bonne Panachee' (a periclinal variegated pear) segregated into pure green and albino plants. 'Red Hardy', a chimeral pear with anthocyanins found in LII only, segregated into pure green and red shoots. The red color was assessed by sugar differential media and a total anthocyanins measurement.

9. Mutation induction

The amount of variation observed *in vitro* may be limited by the amount of variation that preexisted in the parental plant and the mutation rate of the cells *in vitro*. The lack of variability in some cultivars may be overcome by inducing somatic mutations in small plant parts such as leaves or cells with chemicals or irradiation. Mutagens can increase the frequency of variation in culture. The subject has been reviewed briefly by Meins [163] and Skirvin [230] and extensively by Flick [77] and Bourgin [19]. Mutations that occur can be analyzed in the manner discussed earlier.

9.1. *Mutagen-induced chimeras and their separation into pure types*

The phenomenon of chimerism is one of the major obstacles to the use of spontaneous or induced mutations in fruit tree improvement. Chimeras are formed when a somatic mutation occurs within the stratified structure of the apical meristem. Such a mutated apex can give rise to mature tissues of independent origin and different phenotypes. Vegetatively propagated chimeras frequently revert to the non-mutated phenotype. Methods of selecting and

screening fruit crop chimeras have been reviewed by Pratt [185] and discussed earlier.

10. Somatic hybridization

For several years sexual hybridization has been used to improve cultivated crops. However, both intraspecific and interspecific incompatibility have limited the value of the sexual hybridization [66]. The use of cell culture technology has provided a wide potential of using several techniques for crop improvement. For less than 20 years protoplasts have been isolated from many plant genera. The utilization of protoplast fusion in somatic hybridization has been also reported. This technique (somatic cell or protoplast fusion [181], provides a potential for crop improvement. It can be used to overcome incompatibilities that limit successful hybridization, to induce cytoplasmic variability [79,179], to transfer organelles and for genetic transformation [2,65]. For a review of protoplasts and crop improvement see Puite *et al.* [186].

Somatic cell hybridization includes many steps: isolation, fusion, cloning and regeneration of the hybrid [155,183]. The genetic variation, as well as induced variation by a pre-irradiation for cybrid production, may alter the hybridization products. A powerful selection method is necessary to isolate somatic hybrids. Many methods have been described, they vary from general observation to DNA analysis. For instance, Guri and Sink [93] were able to produce a true somatic hybrid between *Lycopersicon esculentum* and *Solanum nigrum*. The hybridity was assessed by both a nuclear genome and a mitochondrial DNA analysis. Fitter *et al.* [76] used monoclonal antibodies which combined with plasma membrane antigens to identify heterokaryons of protoplast fusion.

Very recently Ochatt *et al.* [173b] published a most exciting report of a somatic hybridization of sexually incompatible wild pear (*Pyrus communis* var. *pyraster* L.) and colt cherry (*Prunus avium* × *pseudocerasus*). The hybrid cells regenerated to yield whole plants that were confirmed to be hybrids by morphological features, chromosome complement, and isozyme analysis. The hybrid also exhibits *in vitro* graft compatibility with the both the pear and cherry parents, suggesting its eventual use as a universal rootstock.

Most of the work with protoplasts isolation and fusion has been done with herbeceous plants, mainly from Solanaceae family (for reviews see [2,101,179]. Two examples of herbaceous plants will be discussed. In *Solanum* species, two atrazine-resistant somatic hybrid plants were produced following protoplast fusion between *Solanum melongena* (eggplant) and an atrazine-resistant biotype of *Solanum nigrum* [94]. In another example, a somatic hybrid plant was recovered from a protoplast fusion of *Brassica oleracea* (a cytoplasmic male sterile) and *B. campestris* (atrazine resistant). The genetic analysis showed that the hybrid carried the *B. campestris* chloroplast, while the mitochondria were a combination of both species.

Protoplasts have been isolated from woody plants [3,132], but the ability for protoplast fusion and hybridization is still limited. For instance, Redenbaugh *et al.* [196] were able to isolate protoplasts from cotyledons of different *Ulmus* species but had little success with protoplast fusion except in one species.

In spite of the problems whole plants have been recovered from isolated protoplasts of some woody plants. A new somatic hybrid citrus plant reecently was produced by protoplast fusion between navel orange (*C. sinensis*) and satsuma mandarian (*C. unshiu*) [134]. The hybrid was amphiploid. Similarly, Grosser *et al.* [90] were able to produce and allotetraploid somatic plant from the fusion of 'Key' lime (*Citrus aurantifolia*) with 'Valencia' sweet orange (*Citrus sinensis*) protoplasts. Revilla *et al.* [202] described a simple strategy for the isolation of mesophyll protoplasts from woody species within Betulaceae, Juglandaceae, Rosaceae and Rutaceae families, however, the recovery of plants from the isolated protoplasts was not mentioned. In another example, protoplasts from two *Vitis* spp were succesfully isolated and moved to a culture media. Only callus growth was obtained [147].

In conclusion, somatic hybridization provides a potential for plant improvement, but there are many basic problems to be solved before it will be used regularly.

11. Summary and conclusions

In conclusion, the long generation cycle of tree crops limits the geneticist's ability to make small improvements. However, when a unique plant is obtained, it can be maintained indefinitely by asexual techniques such as grafting, cuttings, layerage, or micropropagation.

The use of tissue culture biotechnologies will facilitate the isolation and identification of improved genotypes for use by the industry and plant geneticists. Initial goals of somaclonal variation programs will include seaches for changes in growth habit, stem and branching habit, flowering and fruiting characteristics. A few years ago, some overly optimistic tissue culturists claimed that the variation available through tissue culture could eliminate the need for traditional plant breeding via crosses and selection pressure. Today, most scientists agree that the variation associated with *in vitro* systems is useful by itself, but it will have even more importance as it is combined with traditional plant breading [204].

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