Section 1

Biology and Biotechnology

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Biotechnology of Trees: Chestnut

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ABSTRACT

Biotechnology has been practiced on chestnuts (*Castanea* spp.) for many decades, including vegetative propagation, controlled crossing followed by testing and selection, genetic and cytogenetic mapping, genetic modification, and gene and genome sequencing. Vegetative propagation methods have ranged from grafting and rooting to somatic embryogenesis, often in coordination with breeding efforts and programs. More recently, particularly in the United States, chestnut biotechnology has included the analysis of genes and genomes with the goal of characterizing and finding disease resistance genes and utilizing them for developing resistant *Castanea dentata* (American chestnut)

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for use in species conservation programs. In this chapter we review chestnut biotechnology, especially with respect to its development in the United States, with emphasis on producing resistant *C. dentata* using various resistant sources of *C. mollissma* (Chinese chestnut) and *C. crenata* (Japanese chestnut).

Keywords: *Castanea dentata*, *C. mollissima*, *Cryphonectria parasitica*, chestnut blight, *Phytophthora cinnamomi*, Phytophthora root rot, disease resistance, backcross breeding, clonal propagation, somatic embryogenesis, genetic mapping, candidate genes, genetic engineering, transgenic, cisgenic

Introduction

The genus *Castanea*, within the family Fagaceae, is an important genus across much of the northern hemisphere's forested ecosystems. *Castanea* contains seven species of deciduous trees and shrubs, classified into three sections—Eucastanon, chestnuts; Balanocastanon, chinkapins; and Hypocastanon, the Henry chestnut (Johnson 1988, Lang et al. 2007). The chestnuts comprises of five species—*Castanea dentata* (Marsh.) Borkh., *C. sativa* Mill., *C. crenata* (Sieb & Zucc.), *C. mollissima* (Blume) and *C. seguinii* (Dode)—are typically most valued and have been most studied especially with respect to disease resistance, genetics of resistance and biotechnology. In Asia and Europe the chestnuts are primarily valued for nut production while in North America they were valued as multi-purpose forest trees providing a wide range of products for local populations through the Appalachian Mountain region. However, in both Europe and North America non-native diseases have limited nut and forest production and decimated the population, respectively (Anagnostakis 1987). Chestnut blight, incited by *Cryphonectria parasitica* (Murr.) Barr, entered the U.S. in the late 1800s, was first detected in 1904 (Merkel 1905; Murrill 1906) and spread throughout the *C. dentata* range (800,000 km^2) by the 1950s, infecting all remaining stands by the 1970s (Beattie and Diller 1954, Hepting 1974). Even prior to chestnut blight, ink disease or Phytophthora root rot, incited by *Phytophthora cinnamomi* Rand, was killing *C. dentata* in the southern part of its range (Crandall et al. 1945, Rhoades et al. 2003). The same diseases are killing *C. sativa* trees in forests and limiting nut production in orchards in Europe, while the Asian species of chestnuts (*C. mollissima* and *C. crenata*) have co-evolved resistance to these diseases as the pathogens are native to Asia (Crandall et al. 1945, Robin et al. 1998).

Biotechnology for mitigating chestnut blight disease has been employed in *Castanea* spp. with the goal of breeding and selecting blight resistant parents for seed production or clonal propagation or developing biological control treatment using hypovirulent mycoviruses of the blight pathogen

(Anagnostakis 1987, Clapper 1952, Dietz 1978, Graves 1950, Hebard 2006, Jaynes 1974, Millgroom and Cortesi 2004). Only more recently has attention increased towards resistance to Phytophthora root rot, in this case using inter-species breeding and selection for resistance in artificial inoculation trials (Jeffers et al. 2009). The common and important feature in these efforts is the presence of natural resistance in the co-evolved host species and their reasonably high interspecies-crossability rates with the highly susceptible non-co-evolved species (Jaynes 1964). In addition, the chestnuts' ability to stump (root collar) sprout after dieback from blight infections (Mattoon 1909, Graves 1926, Paillet 2002) and *P. cinnamomi's* inability to spread into colder environments (Balci et al. 2007, Griffin et al. 2009) have maintained ample germplasm resources of *C. dentata* in the case of North America (Kubisiak and Roberds 2006). In Europe hypovirulence has been effective in slowing and reducing the blight epidemic and the utilization of resistant species as root stocks has enabled the nut orchards to survive and produce (Grente and Berthelay-Sauret 1978, Turchetti 1992). In addition, modern biotechnologies have been and are being employed in these efforts to understand and develop disease resistance (e.g., Barakat et al. 2012, Merkle et al. 2007) and biological control (Dawe and Nuss 2013). These biotechnologies include genome mapping and sequencing, vegetative propagation and tissue culture, and genetic modification of both chestnut trees (e.g., Nelson et al. 2014, Zhebentyayeva et al. 2014) as well as their pathogens and, in turn, their virulence-attenuating viruses (Dawe and Nuss 2013).

The purpose of this review is to provide a summary of the development and application of biotechnologies as they pertain to the efforts to develop resistance in *C. dentata* to chestnut blight and Phytophthora root rot and to restore the decimated ecosystem. Other recent chestnut reviews have focused on silvics (Wang et al. 2013); biological control with mycoviruses (Milgroom and Hillman 2011); integrating technological, ecological and social factors in restoration (Jacobs et al. 2013); vegetative propagation (Viéitez and Merkle 2004); genetic modification for disease resistance (Maynard et al. 2008); and breeding for disease resistance (Worthen et al. 2010).

Vegetative Propagation and Tissue Culture

The ability to propagate *C. dentata* vegetatively has long been recognized as a tool with great potential to aid the restoration of the species. Keys (1978) argued that a practical technique for chestnut vegetative propagation would be highly valuable for multiplying either promising, blight-resistant hybrid genotypes or surviving, possibly resistant *C. dentata*. In addition to clonal propagation of conventionally bred material, vegetative propagation in the form of *in vitro* regeneration of whole plants from single cells is also the only current route for producing genetically engineered plants. Thus, the

availability of at least one reliable *in vitro* propagation system is critical to enable testing of candidate pathogen resistance genes in *C. dentata*.

Macropropagation

Keys (1978) and Viéitez and Merkle (2004) reviewed multiple approaches for chestnut macropropagation, including grafting, rooted cuttings and stool-bed layering. The majority of this research has been performed with Asian and European chestnut species, but some work had been reported with *C. dentata*. Of the different macropropagation approaches applied to chestnuts, grafting has generally been the most successful (Keys 1978, Huang et al. 1994). Incompatibility between the scion and rootstock, particularly with interspecific grafts, has been cited as a problem by some researchers (McKay 1947, Weber and MacDaniels 1969), as has disease susceptibility, but Huang et al. (1994) had an overall success rate of 70% grafting seven *C. dentata* and five *C. crenata* selections onto *C. mollissima* rootstock. The expense of this approach makes it infeasible for mass production of desirable clones (Keys 1978). Nut grafting, which involves removal of the hypocotyl and root from a germinated nut, followed by insertion of the scion into a slit cut into the nut, has been investigated as a less costly alternative to conventional grafting, with some success (Jaynes and Messner 1967). Breeders at The American Chestnut Foundation (TACF) routinely employ the epicotyl budding technique of Ackerman and Jayne (1980) to produce grafts for their breeding orchards, using *C. mollissima* stock, with 10–25% long-term survival (F.V.H., unpublished data).

Rooted stem cuttings would likely be the most practical and least expensive method for mass clonal propagation of chestnuts, but among all woody species, chestnut cuttings, in general, are classified as "very difficult" to root (Wright 1976). Viéitez (1992) reported that cuttings from mature chestnut trees contain chemicals that neutralize the effect of indoleacetic acid (IAA), and thereby inhibit rooting, while juvenile cuttings, which are much easier to root, lack a significant level of these rooting inhibitors. Thus, treatment with rooting hormones is critical to rooting success. The most encouraging results with rooting of *C. dentata* shoots have been with stump sprouts, probably having to do with the juvenility of this material. Jaynes and Messner (1967) reported rooting percentages of up to 75% for some genotypes in a peat:perlite mixture under intermittent mist, following a 1–2 second dip in 5000–8000 ppm indolebutyric acid (IBA). Galic et al. (2014) obtained up to 65% rooting using a similar procedure, but with higher concentrations of IBA (1 or 2%). However, a major obstacle to applying this approach appears to be very low overwintering survival of newly-rooted

stecklings (Keys 1978, Galic et al. 2014). Stool bed layering, which has been used to propagate *C. sativa* and *C. crenata* varieties (Viéitez and Merkle 2004), has also been tested with *C. dentata*, but its success varies widely with clone and it is relatively expensive (Keys 1978).

In vitro propagation

In vitro propagation approaches tested with chestnut have included micropropagation (axillary shoot multiplication), organogenesis (adventitious shoot production) and somatic embryogenesis. As much of the recent work in *C. dentata* propagation and gene transfer have involved somatic embryogenesis, advances made using this approach will be emphasized here.

Micropropagation

By far, the most work on *in vitro* chestnut propagation has been published on micropropagation and the majority of this work has been with European chestnut (see review by Viéitez and Merkle 2004). Early reports of *C. dentata* chestnut micropropagation involved the production and proliferation of axillary shoots by culturing zygotic embryo or seedling nodal explants on medium supplemented with BA, followed by rooting of the elongated axillary shoots (McPheeters et al. 1980, Keys and Cech 1982, Serres et al. 1990). Micropropagation of mature *C. dentata* trees (Read et al. 1985) was accomplished using nodal segments of softwood shoots forced from suckers and branches collected during the winter, cultured on WPM with 0.5 mg/l benzyladenine (BA) to stimulate shoot proliferation. Excised shoots dipped in 3000 mg/l IBA and stuck in Lloyd and McCown's (1980) woody plant medium (WPM) with activated charcoal or sterile sand under high humidity rooted at 40–50%. More recently, Xing et al. (1997) used a similar procedure to produce shoots from a mature *C. dentata*, tree which then underwent a three-step medium sequence whereby shoots were first elongated in a medium with BA, then excised, dipped in IBA and stuck in a rooting medium with activated charcoal and finally returned the same shoot elongation medium for "post-rooting cultivation" to reduce shoot tip necrosis. More recently, Oakes et al. (2013) enhanced rooting percentage and plantlet survival by manipulating exposure to light, activated charcoal and length of time on rooting medium before transfer to post-rooting medium.

Organogenesis

Adventitious shoot production from zygotic embryo axis or seedling explants (epicotyl, hypocotyl, nodal or internodal segment, cotyledonary node) has been reported for European and hybrid chestnuts (reviewed in Viéitez and Merkle 2004), in some cases followed by rooting to produce plantlets, but this regeneration pathway has not been reported for *C. dentata*. However, production of *C. dentata* plantlets from shoots that are apparently adventitious in origin from somatic embryos has become a highly useful method of plantlet production (see below).

Somatic embryogenesis

Over the past 20 years, somatic embryogenesis technology has not only been developed to the point of constituting a viable propagation system, it has come to have a major role in research focusing on *C. dentata* restoration. Repetitively embryogenic cultures capable of producing somatic seedlings were reported for *C. sativa* x *C. crenata* hybrids (Viéitez 1995) and for *C. sativa* (Sauer and Wilhelm 2005). Repetitively embryogenic *C. dentata* cultures were reported by Merkle et al. (1991), although no plantlets were regenerated. In this and subsequent reports (Carraway and Merkle 1997, Andrade and Merkle 2005), embryogenic cultures were initiated from immature seeds dissected from nuts collected in August, and cultured on a semisolid modified WPM with 2 or 4 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). To date, immature seeds have been the only type of explant from which *C. dentata* embryogenic cultures have been initiated. However, Corredoira et al. (2003) initiated embryogenic cultures from leaf explants excised from *in vitro*-grown shoot cultures of *C. sativa*, indicating that a similar approach may be successful with *C. dentata*.

Although somatic embryo germination and some somatic seedling regeneration was reported by Carraway and Merkle (1997), Xing et al. (1999) and Robichaud et al. (2004), conversion (plantlet production) rates remained very low until a suspension culture-based system was developed by Andrade and Merkle (2005). In this system, relatively synchronous populations of somatic embryos were obtained by size fractionating embryogenic suspension cultures, grown in shaken flasks, on stainless steel sieves to obtain small embryogenic cell clumps that were subsequently collected on nylon mesh and plated on basal WPM. Higher percentages of somatic embryos produced in this manner converted than did embryos produced on semisolid medium and the application of at least 12 weeks of cold (8°C) pre-germination treatment and the addition of activated charcoal to the germination medium raised conversion rates to over 70% for some genotypes. The recalcitrance of *C. dentata* somatic embryos to

complete germination, particularly with regard to radicle elongation to produce a taproot, led to the development of an alternative protocol for plantlet production from somatic embryos, in which adventitious shoots were induced from cotyledonary-stage somatic embryos by culturing them on medium with BA and naphthaleneacetic acid (NAA) (Xing et al. 1999, Maynard et al. 2006). Once microshoots were formed, they were multiplied via axillary shoot proliferation and the resulting shoots were rooted following protocols described in Xing et al. (1999), Maynard et al. (2006) and Oakes et al. (2013). This approach has been incorporated into the *Agrobacterium*-mediated chestnut transformation protocol described below.

The availability of highly-productive embryogenic cultures has opened up a number of avenues of research and technology to apply in *C. dentata* restoration work, including gene transfer (see section on Genetic Modification), cryostorage, clonal propagation of elite conventionally bred hybrid genotypes and application of bioreactor technology. Embryogenic *C. dentata* cultures were found to be very amenable to cryostorage, enabling conservation of chestnut germplasm for indefinite time periods. By pretreating cultures with 0.4 M sorbitol and storing them in liquid medium supplemented with 5% DMSO as cryoprotectant, Holliday and Merkle (2000) stored cultures in liquid nitrogen with re-growth frequencies of 95 percent following recovery from cryostorage.

The somatic embryogenesis protocol has been applied to initiate cultures of dozens of *C. dentata* genotypes, including crosses between large surviving *C. dentata* and advanced generation backcross hybrids (B_3-F_3) from TACF's breeding program. The somatic embryogenic protocol, which was originally developed using pure *C. dentata* seeds as explants, gave embryogenesis induction frequencies for open-pollinated TACF B_3-F_3 seed explants (Fig. 1) that were not significantly different from those obtained with pure *C. dentata* seeds, and dozens of B_3-F_3 somatic seedlings (Fig. 2) have already been regenerated (Nelson et al. 2014). Recently, control-pollinated TACF B_3-F_3 seeds also were used to initiate embryogenic cultures, although induction frequencies were not as high as with open-pollinated seeds (Merkle et al. 2013). The ability to clone B_3-F_3 material via embryogenesis will facilitate clonal testing of B_3-F_3 material for blight resistance and other traits.

The combination of hybrid breeding, somatic embryogenesis and cryostorage provides a powerful approach for the production of elite, blight-resistant *C. dentata* clones for restoration and perhaps, eventually, for commercialization. Somatic seedlings derived from cultures initiated from crosses between the best parents could be rigorously field tested while the cultures are held in cryostorage. Once the best clones are identified, those clones could be recovered from cryostorage and scaled-up for mass somatic seedling production.

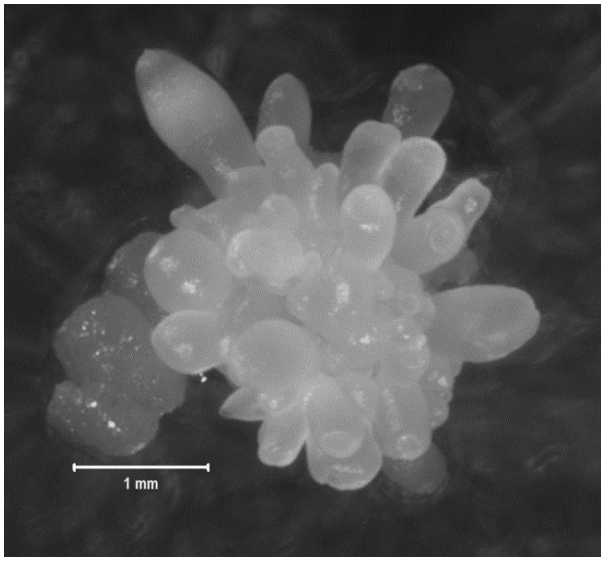

Figure 1. Newly initiated embryogenic culture derived from B_3-F_3 seed explant. Bar = 1 mm.

Figure 2. B_3-F_3 somatic seedlings in the greenhouse.

Recently, the productivity of embryogenic suspension cultures, for both propagation and gene transfer purposes, was further enhanced by the application of airlift bioreactors, which employed a simple construction (1000 mL Kimax bottles with liquid medium, aerated by a vacuum pump). Compared to shaken flasks, bioreactors generated higher yields of tissue

mass and larger fractions of tissue consisting of small cell clumps (< 1 mm in diameter) that were suitable targets for transformation. Bioreactor-generated tissue demonstrated high mature embryo yields and high amenability to transformation via *Agrobacterium* co-cultivation (Kong et al. 2011).

Cytogenetics and Genetic Mapping

An early report concluded that chestnut had $2n = 2x = 22$ (Wetzel 1929) chromosomes. In the following year Jaretzky (1930), through an extensive cytological analysis, reported that the diploid chromosome numbers of *C. sativa* and *C. dentata* Borkh were 24. Later, Almeida (1947) reported that the diploid chromosome number in *C. crenata* was 24. From somatic root tip preparation counts Poucques (1950) reported $2n = 24$ for the diploid chromosome number of *C. mollissima* and *C. dentata*. Jaynes (1962) conducted an extensive cytological study of ten species of *Castanea* where he used the root tip meristems to prepare chromosome spreads, concluding that the haploid chromosome number of *Castanea* was 12 and the diploid number $2n = 2x = 24$.

Recently, high quality somatic root tip mitotic metaphase chromosome spreads of *C. dentata* and *C. mollissima* have been reported using cell wall degrading enzymes (Islam-Faridi et al. 2009). The chromosomes are mostly metacentric and sub-metacentric; and most are in unifocal positions (Fig. 3a). The chromosome spreads proved to be largely free of cell walls, nuclear membranes and cytoplasmic debris, allowing for efficient probe hybridization during fluorescent *in situ* hybridization (FISH). Fluorescent *in situ* hybridization is an important molecular cytogenetic technique for localizing, assigning and orienting genetic markers to specific chromosomes (Heslop-Harrison 1991, Leitch and Heslop-Harrison 1992, Leitch et al. 1992) thereby facilitating the development of chromosome-specific karyotypes

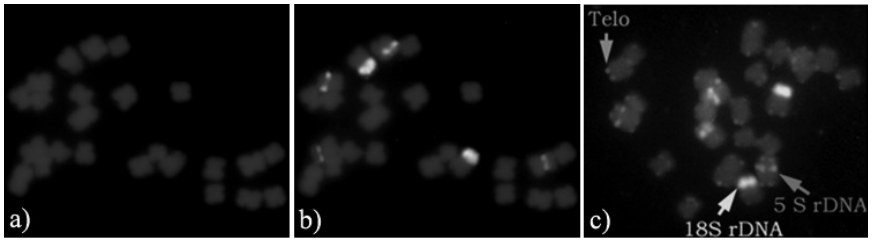

Figure 3. Somatic root tip chromosome spread of *Castanea dentata* and fluorescent *in situ* hybridization with 18S–28S rDNA, 5S rDNA and *Arabidopsis*-type telomere repeat probes; a) chromosome spread counter-stained with DAPI, b) chromosomal location of 18S–28S rDNA (green signals) and 5S rDNA (red signals), and c) each chromosome end showing telomere repeat signals (red signals, orange arrow).

Color image of this figure appears in the color plate section at the end of the book.

and the study of genome organization. Fluorescent *in situ* hybridization with ribosomal gene families (18S–28S rDNA and 5S rDNA) provides excellent cytological landmarks for karyotyping and studying the relationships between species and genera.

The 18S–28S rDNA and 5S rDNA loci were recently located in *C. dentata* and *C. mollissima* using FISH (Islam-Faridi et al. 2009). Two 18S–28S rDNA (one major and the other a minor) and one 5S rDNA sites in both species were identified, and the three sites were located on three different chromosomes (Fig. 3b). The authors also reported that the major 18S–28S rDNA bearing chromosome of *C. mollissima* is structurally different from that of *C. dentata*. In addition FISH signals from the Arabidopsis-type of telomere repeat sequence were observed at the distal ends of each chromosome (Fig. 3c). Similar rDNA loci results have since been reported in *C. sativa* and *C. crenata* (Ribeiro et al. 2011), although different from *C. mollissima* in that two 5S rDNA sites were observed. Interestingly, another difference observed was that the major 18S–28S rDNA was located interstitially and very close to a centromere and co-localized proximally with one of the 5S rDNA sites.

Genetic linkage maps have been constructed for QTL mapping and to facilitate chestnut breeding efforts. An early report (Kubisiak et al. 1997) showed an incomplete map of 11 linkage groups (LGs) in an inter-species F_2 mapping pedigree instead of the expected 12, when analyzed with stringent parameters. This led to a hypothesis that there could be a reciprocal translocation (i.e., exchange of chromosomal arms between two non-homologues) between the two species (*C. mollissima* and *C. dentata*). Almeida (1947) reported noticeable meiotic abnormality in a putative hybrid between *C. sativa* x *C. crenata*. Through an extensive meiocyte study, Jaynes (1961, 1962) reported male sterility in various inter-specific hybrids including *C. sativa* x *C. mollissima*, *C. crenata* x *C. dentata*, *C. dentata* x *C. mollissima* and *C. mollissima* x *C. dentata*. Recently a quadrivalent configuration of a reciprocal translocation has been confirmed in a meiocyte analysis of *C. mollissima* x *C. dentata* (N.I-F., unpublished data). Efforts are being carried out to identify which two non-homologues are involved in this reciprocal translocation and determine what impact it may have on backcross hybrid breeding.

Several genetic maps have been constructed using crosses within and between a few chestnut species. The first map was developed using an inter-species three-generation F_2 pedigree involving a single *C. mollissima* grandparent, two *C. dentata* grandparents, two half-sib related F_1 parents and their F_2 progeny set (Kubisiak et al. 1997). The map proved useful in delineating three QTLs for chestnut blight resistance (*Cbr*1, *Cbr*2 and *Cbr*3), which have now been further characterized (Kubisiak et al. 2013) and

substantially sequenced (Nelson et al. 2014 and see below). A genetic map for *C. sativa* was constructed using a full-sib family (Casasoli et al. 2001) and then upgraded and used in comparative mapping with *Quercus robur* (Barreneche et al. 2004, Casasoli et al. 2006). In addition these two base maps (Kubisiak et al. 1997, Casasoli et al. 2001) were aligned by analysis of common markers (Sisco et al. 2005). Recently an improved (higher density and resolution) genetic map has been constructed for *C. mollissima* (Kubisiak et al. 2013) using two full-sib families. This map has nearly 1400 markers (SSRs and SNPs) based on transcribed sequences and when combined with other genomic resources (i.e., chestnut physical map (Fang et al. 2013), the peach genome sequence, and chestnut genome sequencing (see below) has proven to be useful for candidate gene identification (Nelson et al. 2014).

In addition to genetic maps, a high-quality physical map was made for *C. mollissima* (Fang et al. 2013). Two artificial bacterial chromosome (BAC) libraries with a total of 24X genome coverage were set up and HICF fingerprinted, producing a physical map containing 126,445 clones in 1,377 contigs. A total of 1,026 of the chestnut markers from the *C. mollissima* genetic linkage map were anchored onto the physical map by overgo hybridization. This integrated genetic/physical map framework provides a powerful tool for high resolution trait mapping and sequencing of important genomic regions.

The physical map is now being used to identify BACs for cyto-molecular mapping using FISH. Currently all 12 LGs have been assigned to the 12 chromosomes of chestnut (N.I-F., unpublished data). The major 18S–28S rDNA locus was located on LG_H (Islam-Faridi et al. 2013) while the 5SrDNA locus was located on LG_E which confirmed an earlier result obtained with genetic mapping a 5S rDNA marker (Sisco et al. 2005). A standard karyotype and cyto-molecular map are being constructed using genetically and physically mapped BAC clones (Kubisiak et al. 2013, Fang et al. 2013).

Molecular Markers in Breeding for Blight Resistance

Uses for molecular markers in breeding are almost unlimited and the potential has been beguiling since the days of isozymes. The ultimate goal remains selecting directly for genes of interest, but there are numerous additional applications that rely on less precise knowledge of the genome. A number of those applications have already been made in breeding *C. dentata* for blight resistance and more are in progress. Future efforts will entail next generation sequencing, ultimately to achieve selection directly for, or, equally importantly, against genes of interest.

Current applications

Molecular markers have enabled determination of the recombinant size of the chestnut genome (about 700 cM, Kubisiak et al. 1997, and 800 cM, Kubisiak et al. 2013) and rates of recombination per chromosome. For example, in *C. mollissima* x *C. dentata* F_1 hybrids, about one-half of chromosomes were recombinant per meiosis (T.L. Kubisiak, P.H. Sisco and F.V.H., unpublished data). These determinations have helped optimize progeny sizes and breeding methods (Hebard 2002). As discussed above, the number of major quantitative loci for blight resistance has been estimated at three (Kubisiak et al. 1997, 2013), which has helped optimize progeny sizes and reaffirmed choice of the backcross method of breeding (Hebard 2004).

Marker-Assisted Selection for Recurrent Type (MASRT)

In backcross progenies where mapped marker information existed, MASRT was performed (T.L. Kubisiak, P.H. Sisco and F.V.H., unpublished data). Recovery of recurrent type is the primary reason for the choice of the backcross method of breeding. Marker-assisted selection for recurrent type accelerates recovery of the recurrent type, potentially enabling one to skip generations of backcrossing, which is especially useful in breeding of plants with long generation times, such as chestnut. For TACF's most advanced backcross lines, it is too late for application of MASRT, but the less advanced lines could still benefit.

One recent suggestion is to practice MASRT over two generations (J. Romero-Severson, personal communication), a backcross followed by a filial cross, matching backcross parents of the filial cross to maximize recovery of recurrent type. A potential drawback to this suggestion might be difficulties in obtaining progeny when attempting filial crosses after a wide cross at early stages of backcrossing, such as making B_1-F_2s from *C. mollissima* x *C. dentata* B_1s. In practice, it has been difficult to make F_2s from early generation *C. mollissima* x *C. dentata* F_1s. Segregation distortion would be expected in early generation progeny after a wide cross, making inferences of Mendelian properties much more difficult. Abundant instances of segregation distortion were observed in a *C. mollissima* x *C. dentata* F_2 (Kubisiak et al. 1997) and led to the choice of a pure species cross for the reference mapping population (Kubisiak et al. 2013). These drawbacks led Charles Burnham and Lawrence Inman to oppose making F_2s until the third backcross was in hand (F.V.H., personal information). Presumably, the drawbacks were the reason for departure from the conventional backcross method (Burnham et al. 1986), which calls for an F_2 generation after the initial hybridization, as well as an F_2 generation at B_3 and B_6 (Allard 1960). Traditionally during backcrossing, plants have been selected for recurrent

type using morphological and other macroscopic traits. Molecular maps have helped inform selections based on those traits by illuminating to which linkage groups they map (Kubisiak et al. 1997).

Characterization of genetic diversity

Molecular markers have been used to characterize the genetic diversity of *C. dentata* (Huang et al. 1998, Kubisiak and Roberds 2006, Pierson et al. 2007, Stillwell et al. 2003). *C. dentata* overall is typical of obligately outcrossing hardwood species in estimated heterozygosity and mean similarity (Stillwell et al. 2003). Attempts were made in many of these papers to relate population genetic parameters derived from selectively neutral genetic makers directly to traits such as survival and blight resistance, usually less than convincingly. Some of the surveys also attempted to circumscribe the number of breeding sites and *C. dentata* backgrounds that might be required to restore the species with its diversity intact. In practice, those have been limited by the capacity of breeding efforts. However, the baseline data these studies generated on selectively neutral genetic diversity will be useful in comparing the genetic diversity of products of breeding programs to that of the native population. Kubisiak and Roberds (2006) in particular amassed a large set of specimens from populations across the range of *C. dentata*. The specimens were sampled using a standardized procedure, and their DNA is available for further analysis.

Developing Applications

Determine whether resistance from different sources maps to the same QTLs

Mapped markers could be used to determine whether factors from different sources of blight resistance map to similar QTLs. There were efforts in the late 1990s to do this, but they were plagued by inadequate separation of blight resistance classes (T.L. Kubisiak, P.H. Sisco and F.V.H., unpublished data). The rationale for this approach is that, should genes for blight resistance from different sources map to different QTLs, then one would expect them to have different functionality; deploying resistance genes with different functionality would reduce selection pressure against genes avirulent to specific resistance factors. There would be sufficient polymorphisms to repeat these experiments in new progeny sets genotyped with existing SNPs and SSRs developed by Kubisiak et al. (2013). However, in single-generation pedigrees those polymorphisms would not be able to resolve different alleles for resistance at the same locus or cluster.

Improve characterization of blight resistance

The existing SNPs and SSRs developed by Kubisiak et al. (2013) have been used to genotype an extension of the original F_2 mapping population used by Kubisiak et al. (1997). The hope is to better characterize blight resistance, narrow QTL intervals, detect recessive genes for blight resistance, and detect QTLs with minor effects. Those data currently are being analyzed.

Reduce linkage drag

Linkage drag (Young and Tanksley 1989) is another problem in backcross breeding that could be assisted by the existing marker sets. Presumably, by B_3, most of the donor chromosomes without blight resistance loci would have been discarded. Narrowing the intervals around resistance loci on the remaining donor chromosomes can then be difficult, especially if they are recalcitrant to recombination due to inversions or translocations, for instance. One of the rationales for obtaining B_3-F_2s in a six backcross program is to triple the number of meioses at that generation, thereby increasing the chance of recombination in large blocks of donor genes containing a gene under selection (Allard 1960).

Distinguishing individual plants

Often in breeding, it can be useful to identify individual plants. As was mentioned previously, a very large number of single-nucleotide polymorphism (SNP) and simple sequence repeat (SSR) markers are presently available for chestnut, many with genetic map locations, and some with associations to traits of interest. Utilizing such resources to inform breeding remains a challenge, however (Collard and Mackill 2008). Most of the available chestnut markers are SNPs and were mapped using high-throughput platforms impractical for small sample sizes. Two methods have been tested for small scale genotyping with SNPs: the "WASP" web-based allele-specific PCR assay (Wangkumhang et al. 2007) and the "TSP" temperature-switch PCR (Hayden et al. 2009). Neither method showed high sensitivity or specificity.

SSR markers, if care is taken to select those with large enough differences in allele sizes, can be genotyped successfully by electrophoresis on high-resolution agarose gels (White and Kusukawa 1997) or mini-polyacrylamide gels (PAGE) and visualized by staining with ethidium bromide. SSRs and mini PAGE have been used successfully to detect mis-identified grafted

C. mollissima cultivars and to choose cultivar 'Vanuxem' in preference to 'Mahogany' as the parent of a *C. mollissima* x *C. dentata* F_1 tree whose grafted *C. mollissima* ramet is dead.

Understanding inheritance of phytophthora root rot resistance

Unlike blight resistance, which unquestionably is conferred by more than one gene, resistance to Phytophthora root rot, incited by *P. cinnamomi,* appears to be conferred by a single gene in some populations (Zhebentyayeva et al. 2014). It also has high heritability in hybrids and pure species of *C. sativa*, *C. crenata,* and *C. mollissima* (Fernandez-Lopez et al. 2001). Hence it may be a reasonably straightforward proposition to identify this allele with a usefully high degree of certainty using the genomics tools currently available. A large set of progeny from a single B_1 cross has been generated and screened for Phytophthora root rot resistance that should facilitate this effort.

Future applications

Detecting the alleles for blight resistance with a fairly high degree of certainty

As suggested in the previous paragraph, identification of the alleles that confer blight resistance will be much more difficult than identification of the postulated single allele for Phytophthora root rot resistance, because multiple genes are involved compared to a single factor, each contributing partially to a single measured trait. Genotyping-by-sequencing (see below), either by restriction site associated DNA sequencing (RAD-Seq, Poland and Rife 2012) or sequence capture (Zhou and Holliday 2012), offers the benefit of saturating the gene space relatively parsimoniously. Combined with multiplexing barcoded tags, the procedures are reasonably efficient and inexpensive.

The American Chestnut Foundation and the Southern Institute of Forest Genetics (SIFG) currently have available DNAs from thousands of B_3-F_2s generated by open pollination and their parents. Additionally, the B_3-F_2s have been rated for blight resistance. Using this resource, it should be possible to detect several loci for blight resistance and to position them reasonably precisely. Precise positioning will lessen the number of candidate genes for blight resistance that need to be transformed into chestnut to confirm their activity. The reduced number of candidate genes may allow transformation of all combinations of candidate genes; such combinations may be critical to conferring high levels of blight resistance to the transformants, especially employing cisgenes from within *Castanea*.

Marker-Assisted Selection for Disease Resistance (MASDR)

The American Chestnut Foundation makes B_3-F_2s from within a source of blight resistance to begin eliminating alleles for susceptibility to blight derived from the *C. dentata* recurrent parent. Complete elimination would render the B_3-F_2s true breeding for blight resistance. Direct inoculation of B_3-F_2s cannot be used to distinguish trees with one or two alleles for susceptibility from those with none; rather, some B_3-F_2s must be progeny tested, which also may not lead to elimination of all alleles for susceptibility to blight. Progeny testing is a daunting task. Currently, it is estimated that direct inoculation of B_3-F_2s will enable a reduction in their initial numbers from 27,000 to 1,000, but that reducing the 1,000 to the desired 180 will require progeny testing. The size of the task is also doubled because there are currently two sources of blight resistance. The American Chestnut Foundation will be able to accomplish the task at its professionally staffed breeding station but the breeding program is being replicated at most of TACF's 16 state chapters, which are staffed by volunteers. The volunteers may have more difficulty accomplishing large progeny tests than fulltime professionals. Marker-assisted selection for disease resistance offers the hope of helping select trees homozygous for blight-resistance factors, relieving some of that burden. However, should a low degree of synteny exist between backcross progeny derived from different parents, it may pose a problem for MASDR, especially translating results from one source of blight resistance to another.

Other applications

An ultimate goal for studies of disease physiology in chestnut is elucidation of the molecular basis for blight and Phytophthora root rot resistance. Identifying genes for disease resistance and genes for pathogenicity and virulence in the parasite would be a starting point in this elucidation, not the ending point. Whether or not that molecular basis could be elucidated without reference to a model pathosystem in a plant such as Arabidopsis is unclear. Even with a model pathosystem, success would depend strongly on how well the model system mimicked the tree system. A model system for necrotrophic canker diseases may not exist.

In addition to detecting candidate resistance genes, GBS data can detect and follow all genetic variation segregating in progenies. Careful analysis of the growth and development of backcross chestnut trees in forest progeny tests of B_3-F_2 parents should provide numerous insights into ecologically important aspects of the chestnut genome. Such tests have been in progress since 2009 (Clark et al. 2012). Genotyping-by-sequencing is more efficient with a high-quality reference genome (Poland and Rife 2012, Tennessen

et al. 2013), the lack of which has limited application of GBS in most tree species to date.

Finally, on a more translational level, chestnut could be well served by thorough taxonomic analysis at the molecular level. This has been accomplished to a large degree for chloroplast genomes (Shaw et al. 2012), but taxonomic research using nuclear sequence has not been published yet.

Genome Sequencing and Gene Discovery

Large scale projects have been undertaken to create transcriptome and genome resources for chestnut. The goal of these genome resources projects is to develop high-quality reference sequence databases for use by the greater scientific community for the discovery of genes controlling traits of interest and for marker-assisted breeding. In the U.S., the focus of chestnut genomics resources has been for the discovery of genes related to the chestnut blight fungus, *C. parasitica*.

Transcriptome sequence databases for *C. mollissima* and *C. dentata* were developed in the "Genomic Tools for the Fagaceae" Project sponsored by the National Science Foundation's (NSF) Plant Genome Research Program (PGRP). Ten RNA samples were prepared from various tissues of *C. mollissima* and *C. dentata*, including blight-infected stem, uninfected stem, and whole plant (combined stem, leaf, catkin, and bud). Tissues were sampled from *C. mollissima* genotypes 'Nanking' and 'Mahogany'. *Castanea dentata* genotypes 'BA69', 'Wisniewski', and 'Watertown' were selected for tissue sampling from breeding stock of The American Chestnut Foundation and the Connecticut Agriculture Experiment Station.

The ten RNA samples were converted to cDNA libraries and sequenced using the high throughput 454 sequencing platform. A total of 172Mb and 214Mb of raw sequence data (reads) were obtained for *C. mollissima* and *C. dentata*, respectively. The reads were assembled into a unigene set, i.e., a reconstructed set of unique transcripts found in each species. For *C. mollissima*, the final unigene set contains 48,335 putative unique transcripts with an average length of 537 bases, and for *C. dentata*, the unigene set contains 45,288 transcripts with an average length of 449 bases (Barakat et al. 2009).

In addition to a reference set of transcript sequences, the reads from the individual libraries indicate the relative expression levels of genes in each tissue. This may be used to detect patterns across gene networks and pathways that are increased or decreased in expression between experimental conditions. Barakat et al. (2009) reported on the differential gene expression patterns between the blight-induced canker tissue versus the healthy stem tissue within the *C. mollissima* and *C. dentata* cDNA libraries. The study

found that both species of chestnut utilize local and systemic resistance response genes to fight *C. parasitica* infection and activate physical/chemical blocking mechanisms through the hypersensitivity response and lignin synthesis, an early step in wound periderm formation (Hebard et al. 1984). The reference transcript set and genes known to be differentially expressed in canker versus healthy tissue provided the first set of genes from *C. dentata* and *C. mollissima* for MAS and for further biotechnology applications in inducing resistance to *C. parasitica* in *C. dentata*.

From *C. dentata* and *C. mollissima* transcriptome resources, thousands of simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs) were identified, which may be used in a variety of genetic analyses such as linkage mapping, genetic association studies, population genetics, and marker-assisted selection. These transcriptome-based DNA markers found immediate application in the construction of a high density, high-resolution genetic map for *C. mollissima* containing 329 SSR and 1,064 SNP markers (Kubisiak et al. 2013, described above). The genetic map provides the most comprehensive map for a *Fagaceae* species to date and will be invaluable in comparative genomics and gene discovery research.

The availability of a reference genome sequence—the full complement of coding and noncoding DNA in an organism—increases the efficiency and accuracy of genetic and biotechnology efforts (Wullschleger et al. 2013). While reference genomes exist for many plants, there are no species with a reference genome in the order Fagales. In 2009 a genome sequencing project for chestnut was initiated with support from the Forest Health Initiative, a collaborative effort of the U.S. Endowment for Forestry & Communities, the USDA Forest Service, and Duke Energy (www.foresthealthinitiative.org, Nelson et al. 2014). The important choice of species and genotype to serve as the reference genome for chestnut was straightforward. *Castanea mollissima* is the primary source material being used to transfer resistance to the chestnut blight fungus *C. parasitica* and the ink disease-causing oomycete *P. cinnamomi* to *C. dentata* by the long-standing, multi-generational back-cross breeding program conducted by TACF. The cultivar 'Vanuxem' was chosen for the reference genome due to the key role it played in the TACF breeding program and in the construction of the genetic and physical maps described above.

The objectives of the *C. mollissima* genome project are to provide the research community with a reference chestnut genome to identify all the genes in the three blight resistance QTL and as a source of gene discovery for use in transgenic and molecular breeding approaches to blight-resistant chestnut. It will also be useful for studying the genome evolution that occurred after the divergence of the individual chestnut species and as a comparative genome for use in other related Fagaceae species such as oak and beech.

The chestnut genome project is ongoing, but extensive data production and initial assemblies have already been performed. The sequencing approach relies on "Next Generation" DNA sequencing technologies. To date over 61 billion bases of genomic DNA sequence data have been produced, utilizing both Illumina MiSeq and 454 Life Sciences platforms. This represents 76X coverage of the estimated 800 Mbp genome (Kremer et al. 2007). The current assembly version includes 724,428,616 bp in 41,270 scaffolds,[1] with an N50[2] scaffold length of 39,580 bp. The scaffolds were built from 323,611 contigs covering 843,288,101 bp, with an N50 contig size of 9,473 bp. The current genome assembly provides good overall coverage of the 800 Mb chestnut genome.

Because of heterozygosity in chestnut, a complete assembly may require much more than the 1C genome size to be covered. Using the current data the Newbler assembly software (454 Life Sciences, Branford, Connecticut) estimates a complete assembly size of 1,844.3 Mb, approximately twice the 1C genome size. The chestnut genome project is continuing to produce genome sequence to improve the assembly. In addition, the sequence-based genetic markers from the recently published genetic linkage map and physical map are being used to align as many of the scaffolds in the current assembly to chromosomes and linkage groups as possible.

The current genome assembly was structurally and functionally annotated using the *C. mollissima* and *C. dentata* unigene transcript sequences and the proteomes of peach (*Prunus persica*) and *Arabidopsis thaliana*, yielding a minimum of 38,146 predicted genes. Additional annotations based on gene sequences from other reference plant genomes yielded a slight higher total of 38,268 genes. The number of genes predicted per scaffold ranged from 0 to 31, with an average of 4.6 exons per gene. The total number of genes is within the range of genes found in other sequenced plant genomes such as *Arabidopsis thaliana* with 27,416 genes, *Glycine max* with 54,175 genes, *Prunus persica* with 27,416 genes, *Populus trichocarpa* with 41,335 genes, and *Vitis vinifera* with 26,346 genes (Goodstein et al. 2012). The chestnut annotation will be updated as new whole genome sequence assemblies are released.

To identify all of the potential blight resistance genes, BACs from the *C. mollissima* physical map covering the three major blight resistance QTL were sequenced using the Illumina MiSeq technology. The QTL sequences were assembled into a total of 2,291 contigs covering 14.4 Mbp of genome sequence. The contigs were ordered across 395 scaffolds,[1] covering 13.9 Mb,

[1] Scaffold versus contig. During a genome assembly, contiguous pieces of reassembled bases are known as contigs. Using positional information, contigs can often be ordered with gaps of unknown bases (Ns) in between, creating a longer "scaffold".

with an average length of 35 Kb and an N50 length[2] of 86 Kb. Over 700 genes were identified in the assembled QTL sequences, of which 22 were selected as high priority candidate genes for blight resistance. Many are part of the response pathways originally identified in the transcriptome sequencing of canker and healthy stem tissues.

All of the genomic resources for *C. mollissima* and *C. dentata* including the unigenes, genetic markers, genetic map and physical map are publicly accessible through the Fagaceae Genomics Web (http://www.fagaceae.org). The genome sequences and interactive browser for viewing and searching the assembled genome sequence will be available at the Hardwood Genomics Project website (http://www.hardwoodgenomics.org/chinesechestnut), along with links to the transcriptome resources and related sites. A website for the chestnut genome project has also been initiated at the National Center for Biotechnology Information (NCBI) to house the data and facilitate access to the final results (http://www.ncbi.nlm.nih.gov/genome/10727).

Genetic Modification

In its broadest definition, genetic modification of chestnut to produce blight-resistant trees began as early as 1922 with production of *C. dentata*/*C. crenata* hybrids (Detlefsen and Ruth 1922) and is continuing today using a variety of *Castanea* spp. and hybrids, as discussed earlier in this chapter. Another type of genetic modification, mutational breeding, has been used to produce over 2500 varieties of crops since the 1920's and was first tried on American chestnut in 1956. This method used an ionizing radiation treatment of the nuts to induce random mutations, then crossed the resulting trees with wild-type *C. dentata*, and began screening for enhanced blight resistance. The surviving M2 and M3 generations are still being studied today (Dietz 1978, Burnworth 2002). Although there are several ways to modify chestnut trees genetically, this section of the chapter will focus on one of the newest approaches, genetic engineering. In this section genetic engineering is defined as transformation of organisms with relatively small sequences of recombinant DNA containing functional genes.

Two terms have recently entered the genetic engineering vocabulary: transgenic and cisgenic. Transgenic is defined as moving genes between unrelated species, while cisgenic refers to moving genes between closely related species that have the capacity to interbreed. 'Intragenic' is sometimes

[2] N50 statistic. A statistic often used for describing a genome assembly. Considering 50% of the bases of the assembly in the largest pieces, it is the smallest sized piece in that set, i.e., 50% of the assembly is in pieces of the N50 size or larger.

used to define genetic engineering where the gene comes from the same species. Some researchers interchange the terms cisgenics and intragenics, but for chestnuts, these two terms have little difference when working with cDNA clones because cDNA clones are often identical or very similar between species. For example, in a comparison of an orthologous laccase-like gene from *C. dentata* and *C. mollissima*, the genes have a 99% cDNA sequence identity and 100% amino acid sequence identity (see below). So in this case, cloning the cisgene or intragene cDNA would not make much difference. However, if one were to clone the genomic form with their promoters, terminators, and introns, the differences would become biologically significant.

Since to date the focus in genetic engineering of chestnut has been on cDNA cloning, only cisgenic and transgenic will be used with respect to the gene's source. So why is there an emphasis on the differences between the sources of a gene? It is mainly because there is a hypothesis that the public will be more accepting of cisgenics than transgenics. This may or may not be true, but trying to promote cisgenics as "safer" might only promote the impression that transgenics are not safe, which is not necessarily true. Transgenics can be as safe as products of any breeding technique. Also, cisgenics is not well defined. For example, if you use a cisgenic cDNA clone, but drive it with a promoter from another species, is it truly cisgenic? In the end, all methods, including traditional breeding, cisgenics, and transgenics have reasonably similar risks and benefits. Genetic engineering of *C. dentata* is currently employing both transgenics and cisgenics. The earliest work began with transgenics because of the availability of putative resistance-enhancing genes from other plants, but with the recent advances in genomics, previously described in this chapter, many resistance-enhancing candidate genes are now available from *C. mollissima* and *C. seguinii* (Table 1). Which genes will eventually be used will depend on effectiveness, safety, and public acceptance. Since the current work includes 33 genes, to simplify the descriptions, one transgene, oxalate oxidase from wheat (*Triticum aestivum*) (Lane et al. 1993), and one cisgene, a laccase-like gene from *C. mollissima* (Baier 2010), will be used as examples.

Oxalate oxidase (OxO) (EC 1.2.3.4) genes have been shown to enhance pathogen resistance in a number of transgenic plant species. Livingstone et al. (2005) and Partridge-Telenko et al. (2011) presented evidence that transgenic peanuts were resistant to *Sclerotinia minor* Jagger. For resistance to *Sclerotina sclerotiorum* (Lib.) de Bary, evidence was presented for soybean (Donaldson et al. 2001), sunflower (Hu et al. 2003), and rape (Dong et al. 2008). Walz et al. (2008) presented evidence for resistance of transgenic tomato to *Botrytis cinerea* (De Bary) Whetzel. Schneider et al. (2002) presented evidence that transformation of potato with OxO genes conferred resistance to *Phytophthora infestan*s (Mont.) de Bary and

Table 1. Genes currently being tested in *C. dentata.*

Transgenes	**Cisgenes***
Oxalate Oxidase (wheat)	Laccase-like protein/diphenol oxidase (Cm)
Stilbene synthase (grape)	Ethylene Transcription Factor (Cm)
NPR1 (Arabidopsis)	Proline-Rich Protein (Cm)
Gastrodia anti-fungal peptide	Deoxy-arabino-heptulosonate phosphate synthase (Cm)
CaAMP (pepper)	Thaumatin-like protein (Cm)
ESF39 (designed AMP)	Lipid transfer protein SSH (Cm)
	Acid Phosphatase (Cm)
	CBS domain containing protein (Cm)
	Beta 1,3 Glucanase (Cm)
	Myo inositol phosphate synthase (Cm)
	Lipid transfer protein/proteinase inhibitor (Cse)
	Caffeoyl-CoA-O-methyltransferase (Cm)
	Cystatin, cysteine protease inhibitor (Cm)
	Resistance to *Phytophthora* (Cm)
	Non-expressor of Pathogen Response 3/4 (Cse)
	Peroxidase (Cm)
	Subtilisin-like protease (Cse)
	UDP-glycosyltransferase (Cm)
	Malic enzyme (Cse)
	Cinnamyl alcohol dehydrogenase-like protein (Cm)
	Shikimate dehydrogenase (Cm)
	Phenylalanine ammonia-lyase (Cse)
	ACC oxidase (Cm)
	Glucan endo-1,3-glucosidase (Cm)
	Cytochrome P450 allene oxide synthase (Cse)
	Glutathione s-transferase (Cm)

**Castanea mollissima* (Cm), *Castanea seguinii* (Cse)

to *Streptomyces reticuliscabiei* Bouchek-Mechiche et al. (2006), but the OxO gene did not enhance resistance to *Erwinia carotovora* (Jones 1901) Bergey et al. 1923, which was also tested. He et al. (2013) presented evidence for resistance of transgenic taro to *Phytophthora colocasiae* Racib. In addition, Liang et al. (2001) found that transformation of hybrid poplar with OxO genes conferred resistance to *Septoria musiva* Peck. These pathogens include fungi, oomycetes, and bacteria, but other pests or abiotic stresses may be addressed by OxO as well. Transgenic maize expressing the wheat OxO gene showed altered secondary metabolism that enhanced resistance to an insect pest, the European corn borer (Mao et al. 2007). And finally, the wheat OxO increased tolerance to oxidative stress in transgenic tobacco (Wan et al. 2009). Oxalate oxidase breaks down oxalate (or oxalic acid) into carbon

dioxide and hydrogen peroxide. Hydrogen peroxide is involved in plant stress response pathways and can have profound effects on gene expression to protect the plant (Vandenabeele et al. 2003).

Oxalate has been hypothesized for many years to be a major virulence factor used by *C. parasitica* (Havir and Anagnostakis 1983, Powell 1986). More recently it was shown that knocking out the oxalacetate acetylhydrolase gene in *C. parasitica* prevents oxalate production and significantly reduces the pathogen's virulence (Chen et al. 2010), supporting this hypothesis. It was predicted that the OxO gene could enhance blight resistance in *C. dentata* by degrading the oxalate produced by the pathogen (Powell et al. 2006). Early results showed that expression of the OxO gene in transgenic chestnut callus tissue could protect lignin formation in the presence of oxalic acid (Welch et al. 2007). Using a predictive leaf assay (Newhouse et al. 2013), recent results showed that the oxalate oxidase gene could indeed enhance resistance to *C. parasitica* in transgenic *C. dentata* (Zhang et al. 2013). Field trial inoculations of stems in 2012 confirmed leaf assay results and showed that the OxO transgene enhanced blight resistance in *C. dentata* (Nelson et al. 2014). The level of OxO gene expression influences levels of resistance. For example, tissues derived from the 'Darling 11' transgenic event have high levels of OxO expression because the transgene is driven by the CaMV 35S promoter. Trees from this event had even less lesion necrosis on leaf assays than the blight-resistant *C. mollissima* controls (Fig. 4). On the other hand, in the vector used for the 'Darling 4' event, the OxO gene is driven by the VspB promoter. This promoter produces less gene product, with the expression predominately in the vascular tissues. The leaf assays show an intermediate level of lesion necrosis for this event between *C. dentata* and *C. mollissima*, yet it is still significantly different ($p < 0.05$) from the *C. dentata* control (Fig. 4).

In addition to chestnut blight, Phytophthora root rot is a serious disease of chestnut in the southern portion of the tree's natural range. Interestingly, it has been reported that the OxO gene has enhanced resistance to other *Phytophthora* species (He et al. 2013, Schneider et al. 2002) even though these pathogens do not produce oxalate. It may be that the generation of the H_2O_2 byproduct from oxalate catabolism plays a significant role with these pathogens. Assays for resistance to *P. cinnamomi* on the transgenic chestnut will soon be started. Whatever the mechanisms, the OxO gene might provide an opportunity to enhance resistance to multiple pathogens using a single gene.

The laccase-like gene was the first cisgene cloned from *C. mollissima* and used to transform *C. dentata*. This gene was identified by interspecific suppression subtractive hybridization using *C. mollissima* and *C. dentata* canker margin tissues (Baier 2010). The orthologs' amino acid sequences are identical, but this gene is much more highly expressed in the *C. mollissima*.

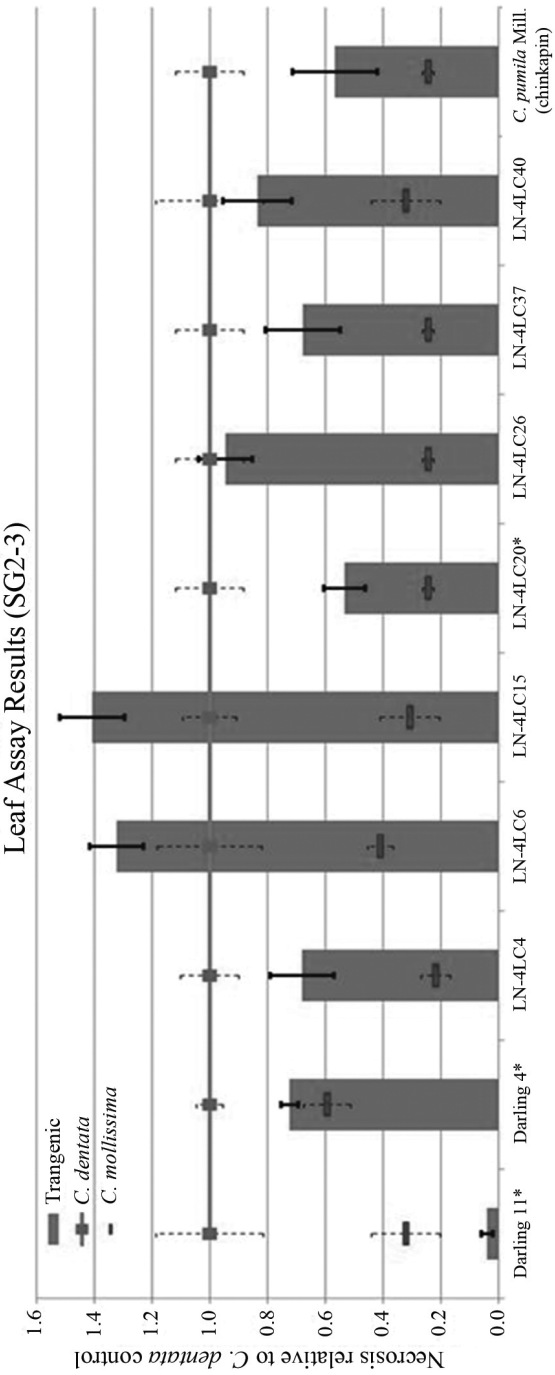

Figure 4. Leaf assay results from laccase-like gene events and controls. Relative necrotic area was measured (LxW). Scale was normalized to blight-susceptible *Castanea dentata* control (blue line and squares). Blight-resistant *Castanea mollissima* controls are represented by small, red, rectangular boxes. The green bars represent the leaves being tested. Additional controls: Darling 11 is an oxalate oxidase (OxO) event with high resistance, Darling 4 is an OxO event with intermediate resistance, and *Castanea pumila* is a related species that has been shown to have intermediate blight resistance. The LN-4LC# are different events expressing the *C. mollissima* laccase-like gene. A "*" denotes necrosis is significantly different than the *C. dentata* (t-test P<0.05) (A. Newhouse and W.A.P., unpublished data).

Color image of this figure appears in the color plate section at the end of the book.

Therefore it is hypothesized that its overexpression will enhance blight resistance. There are multiple ways this gene might be associated with resistance. High concentrations of a hydrolysable tannin (ellagitannin) was associated with blight resistance in callus tissue (Hebard and Kaufmann 1978) and a significant step in ellagitannin synthesis is catalyzed by a laccase-type phenol oxidase (EC1.10.3.2) (Niemetz and Gross 2005). The enzyme laccase is also thought to be involved in cell wall reinforcement through lignification (Dean et al. 1998, LaFayette et al. 1999, Wang et al. 2008). Oxidized phenols also could tan host carbohydrates and proteins, rendering them unavailable as nutrients for *C. parasistica,* which would probably limit fungal growth and development, including that of mycelial fans. A lower rate and extent of formation and expansion of mycelial fans distinguished blight-resistant from blight-susceptible chestnut (Hebard et al. 1984). Lastly, metabolomic studies by T.J. Tschaplinski (T.J. Tshaplinski and W.A.P., unpublished data) showed higher levels of lignans in *C. mollissima* stem tissues than in *C. dentata* that parallel the differences in laccase-like gene expression. Laccases have been associated with lignan synthesis (Tranchimand et al. 2006) and lignans can have antifungal properties (Cho et al. 2007).

Forty American chestnut transgenic events have been produced using the pFHI-CmLac vector constructed by C.J. Nairn (unpublished data). In this vector, the laccase-like gene is driven by the UBQ11 constitutive promoter, and due to insertion position effects, it was possible to select events with different expression levels for leaf assays used to predict blight resistance levels (Newhouse et al. 2013). The leaf assays on the first seven events showed one that, to date, has significantly enhanced pathogen resistance ($p<0.05$, Fig. 4). Improvements in the leaf assay procedure are being evaluated in an effort to detect finer differences between the test plants and controls, which may help detect significant differences between the events with smaller changes in resistance. The results so far indicate the laccase-like gene might enhance pathogen resistance, but only moderately, similar to what is seen in chinkapins that have intermediate levels of resistance (Fig. 4). This is much less improvement than observed with the OxO transgene, but multiple genes or multiple mechanisms of resistance may play a valuable role in a restoration program.

The results to date indicate that the OxO transgene will be more effective at enhancing blight resistance than the laccase-like cisgene. Many more candidate genes are in the queue to be examined. But even if the three or more resistance enhancing cisgene alleles in *C. mollissima* are identified, each one is only part of a quantitative resistance system. Therefore none are expected to provide full resistance alone, and it may be necessary to combine these genes in a pyramid construct. For example, in preliminary tests, the proline-rich protein gene appears to be enhancing pathogen resistance

to intermediate levels, similar to the results with the laccase-like gene. If these results hold up, the next logical step would be to stack these genes to determine if even higher levels of resistance can be obtained. On the other hand, it is feasible that transgenes could provide not only partial resistance, but full resistance. Ultimately, the best approach might be to forget the cisgene/transgene debate and move forward with both. For sustainable blight and *Phytophthora* resistance, it will be necessary to pyramid both types of genes. As with most tree species, producing durable resistance is a key to forest health. Perhaps durable resistance will be achieved by stacking genes that enhance resistance by different mechanisms. Identification of multiple genes will facilitate such stacking.

Conclusion

The genus *Castanea* comprises an ecologically important and economically valuable group of forest trees and nut-producing orchard trees. Their importance and value ranges across much of the northern hemisphere. In both Europe and North America, *Castanea* spp. have been subjected to introduced pathogens that have devastated the native species. Efforts to identify and develop resistance to these diseases have been the focus of much of chestnut biotechnology research over the past 100 plus years. Species hybridization and testing and selection for resistance, vegetative propagation of excellent phenotypes, development of molecular marker and genetic maps to aid selection and breeding efforts, genetic modification using putative resistance genes, and gene and genome sequencing have all been part of this mix. Much progress has been made in each of these areas and recent efforts of integrating the technologies offer much promise for developing resistant genotypes and propagating them at scale such that species reintroduction and ecosystem restoration may commence. For successful restoration it will be important that the resistant genotypes are genetically diverse in background genetics as well as resistance genes, and adapted to various environmental conditions. Continuing cooperation and collaboration among research groups across the northern hemisphere should ensure this outcome. We hope this review, although focused on *C. dentata* and thus North America, will help foster more collaborative research and development on the biotechnology and genetic advancement of *Castanea* spp. world-wide.

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