Review

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Abstract

European chestnut is an agroforest species of great ecological, economic, and cultural importance in many temperate regions. However, in recent decades, it has been seriously threatened by various factors, including devastating diseases such as chestnut blight and ink disease, as well as the impacts of climate change. In this context, biotechnological tools have emerged as a key alternative for the protection, improvement, and sustainable use of the species. This paper analyzes the main biotechnological strategies applied to European chestnut. First, classical and assisted breeding techniques are discussed, including controlled hybridization and the use of molecular markers to accelerate the selection of genotypes of interest. In the field of molecular biotechnology, studies related to the identification of key genes, the development of genetic markers (SSR, SNP), and the omics characterization of chestnut are reviewed. The use of micropropagation techniques for the clonal multiplication of elite individuals is also included. Furthermore, advances in genetic modifications are explored, highlighting the introduction of resistance genes through transgenic and cisgenic approaches, as well as emerging technologies such as CRISPR/Cas9. Finally, future perspectives for the application of biotechnology in the recovery, improvement, and sustainability of chestnut in the face of current and future threats are presented.

Keywords: blight disease; conservation; *Castanea sativa*; CRISPR/Cas9; gall wasp; ink disease; micropropagation; molecular markers; omics

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1. Introduction

Castanea sativa Mill., also known as sweet chestnut or European chestnut, is a tree species belonging to the family Fagaceae. The genus *Castanea* is taxonomically divided into 13 species, the most important of which are *C. sativa*, *C. dentata* (Marsh.) Borkh. (American chestnut), and *C. crenata* Siebold & Zucc and *C. mollissima* Blume (Asian chestnuts) (Figure 1). Palynological studies indicate that the European chestnut was widely distributed during the Tertiary period, retreating to refugia in southern Europe following successive glaciations. Pollen studies also suggest a second, rapid expansion associated with human activity, particularly linked to the expansion of the Roman Empire [1]. This anthropogenic influence likely played a key role in the current distribution of the species across Europe, facilitating its spread beyond natural refugial areas through cultivation and forest management. Today, sweet chestnut is found throughout central and southern Europe including the northern Iberian Peninsula, southern France, central and northern Italy, and the southern Balkan Peninsula as well as Asia Minor, particularly western and northern Turkey and the Caucasus region [2] (Figure 1). European chestnut holds significant ecological, economic, and cultural importance. It is a multipurpose tree valued for its high-quality, durable, and rot-resistant wood, which is widely used in furniture making, fencing, and construction [3]. In addition to its timber, the species produces edible nuts and supports a variety of secondary products such as pasture, mushrooms, and berries, contributing substantially to rural livelihoods and agroforestry systems. The nutritional value of chestnuts has been recognized since ancient times, with some Greek and pre-Roman tribes even considering them superior to almonds and walnuts. According to Vieitez and Merkle [4], Montaigne wrote in his *Journal du Voyage* around 1570 that the Roman legions, during the Gallic Wars, survived thanks to chestnuts, which they referred to as the "bread of the forests" and called the tree *Arbus panis*. Currently, chestnut nuts remain highly important as a key source of income in many rural areas across Europe and are increasingly in demand for gluten-free and healthy food products. Rich in complex carbohydrates, fiber, vitamins, and antioxidants [5], chestnuts are appreciated both in traditional and modern cuisine. Finally, the European chestnut holds significant cultural value in southern Europe, historically connected to local food traditions, landscapes, and customs. It has been a vital source of sustenance during times of scarcity and features prominently in popular festivals such as *Magosto* in Spain, *Magusto* in Portugal, or *Sagra* in Italy. Its presence is evident in rural architecture, traditional cuisine, and agricultural landscapes, symbolizing resilience, sustainability, and a living heritage closely tied to the holistic use of forest resources.

Over the past century, two diseases have severely affected both European and American chestnut, with ink disease being one of the most destructive [6]. First reported in Spain and Portugal in the 19th century, it is now widespread across many European countries. The disease is primarily caused by *Phytophthora cinnamomi* Rands, prevalent in Spain, Portugal, and France, and by *P. cambivora* (Petri) Buisman, more common in Italy and Greece [7]. These oomycetes, whose cell walls contain cellulose and glucans instead of chitin, infect tree roots and spread through living tissues, eventually disrupting the transport of water and nutrients. Symptoms include chlorosis, reduced fruit size, wilting, and ultimately tree death [8]. The disease is identified by a dark necrotic area at the root collar and a black exudate in the surrounding soil. It spreads rapidly through zoospores and chlamydospores in moist soils, with its progression influenced by both environmental conditions and human activity. Its spread has been documented in both plantations and natural forests, and its impact is expected to increase under future climate change scenarios, as predicted by simulation models [8,9]. The distribution of this oomycete has previously been linked to climate conditions [10]. It is strongly limited by low temperatures, becoming inactive in soil below 10 °C and unable to grow in artificial media under 5 °C [11,12]. Although some isolates can produce sporangia at 7.5–10 °C, effective infection of host plants does not occur below 8–9 °C, restricting their presence in alpine and subalpine regions [13]. The distribution of *P. cinnamomi* shown in Figure 1 is based on data obtained from EPPO [14], which includes records from the 1990s for some regions with severe winters (i.e., some northeastern U.S. states; Canada). Therefore, some of these records may reflect temporary introductions that are no longer present and have not been recently reassessed. The other major

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disease affecting chestnut is blight, caused by the fungus *Cryphonectria parasitica* (Murr.) Barr., which infects the aerial parts of the tree through wounds [15]. *C. parasitica*, native to East Asia, was introduced to North America in the late 19th century, where it decimated populations of the American chestnut. It is now widespread in the eastern US and present in parts of Canada (Figure 1). In Europe, the fungus was first reported in 1938 near Genoa (Italy) on European chestnut, and has since spread throughout much of Europe, showing variable distribution and impact across different regions (Figure 1). The fungus invades the cortical tissue and develops as a saprophyte, causing necrosis that can completely girdle branches or the trunk, leading to the death of the affected organ [6]. Although the tree may partially survive, both wood quality and fruit production are severely impacted. In response, the tree produces epicormic shoots at the base of the canker. In European chestnut, the pathogen virulence is reduced due to hypovirulence caused by a hypovirus [16](and references therein); however, in recent years, severe outbreaks have occurred in Spain and northern Portugal [17,18]. Finally, chestnut trees have been increasingly affected by another threat, an insect pest known as *Dryocosmus kuriphilus* Yasumatsu, which causes significant damage, especially when infestations persist over successive years [19]. Native to China, it has spread to major chestnut-growing regions worldwide and is now extended across East Asia, North America, and much of Europe, including Italy, France, Spain, and Portugal (Figure 1). Galls formed by this insect develop on shoots, leaf midribs, or stipules, and following adult emergence, they substantially alter the branch architecture of chestnut trees [20]. This results in up to a 70% reduction in leaf area, fewer dormant buds, and a decline in the production of wood and flowers [21]. Infestation by the chestnut gall wasp also leads to significant reductions in nut yield, with annual losses reported between 15–30% in China and 50–75% in the United States [22]. Interestingly, Asian species such as *C. crenata* and *C. mollissima* are resistant to both ink disease and blight but are susceptible to the gall wasp [22]. The spatial distribution of the three pathogens is presented in Figure 1.

2. Biotechnological Tools Applied to Chestnut

An effective alternative for managing diseases primarily caused by *P. cinnamomi* is the development and deployment of resistant or tolerant chestnut plants, which can subsequently be used for reforestation in affected or susceptible areas. While tolerance could theoretically be achieved through conventional breeding programs, such long-term improvement initiatives have rarely been implemented for chestnut and other long-rotation hardwood species due to the extended timelines involved. As noted by Savill et al. [23], investments in projects with lifespans exceeding 40 years have historically been unattractive, a sentiment echoed by Vieitez et al. [24]. Among hardwoods, chestnut stands out as one of the few species where classical genetic improvement programs targeting *P. cinnamomi* tolerance have been actively pursued. During the early 20th century, hybridization between European chestnut and Asian resistant species (*C. crenata* and *C. mollissima*) represented the primary strategy to combat ink disease. In Spain, this effort was spearheaded by Cruz Gallástegui at the Biological Mission of Galicia (MBG-CSIC) **[25]**. Although the resulting hybrids exhibited enhanced tolerance to *P. cinnamomi*, they generally produced smaller trees and fruits of inferior size and quality compared to pure European chestnut. Currently, in the rainy northern regions of Spain, hybrid rootstocks have helped mitigate the impact of *P. cinnamomi*; however, these rootstocks are less suitable in southern regions due to their lower tolerance to cold and drought conditions [26].

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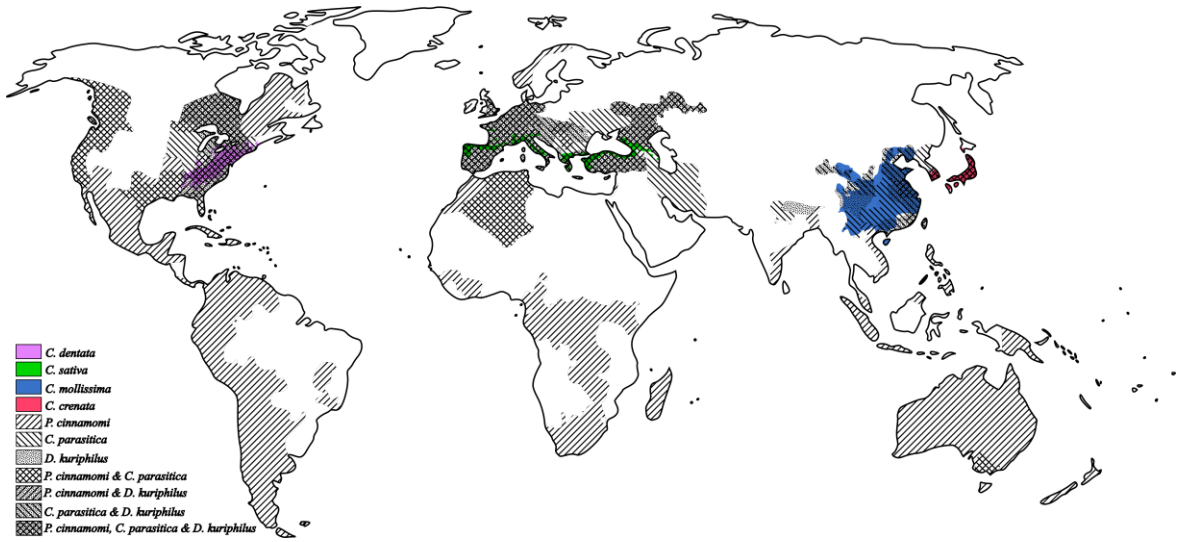

Figure 1. Map illustrating the geographic distribution of natural populations of *Castanea sativa* (European chestnut), *C. dentata* (American chestnut), *C. mollissima* (Chinese chestnut), and *C. crenata* (Japanese chestnut), along with the primary biotic stresses impacting each species, including chestnut blight (*Cryphonectria parasitica*), ink disease (*Phytophthora cinnamomi*), and the chestnut gall wasp (*Dryocosmus kuriphilus*). Map created with data from several sources [14,27–32] using Inkscape [33]. Map background available at https://commons.wikimedia.org/wiki/File:Simplified_blank_world_map_without_Antartica_(no_borders).svg.

Forest biotechnology offers promising tools to accelerate tree improvement by integrating molecular techniques into breeding programs. These include marker-assisted selection, genotyping for individual tree identification, the discovery of genes or alleles linked to pathogen resistance, as well as genetic engineering approaches, in vitro culture techniques, and cryopreservation for the development, propagation, and long-term conservation of improved genotypes [3,34,35]. In particular, genomic selection holds great potential to significantly shorten the time required for genetic evaluation of desirable traits in trees, thereby enhancing the efficiency and precision of breeding efforts [36,37].

Here, we provide a comprehensive review of the various biotechnological methods applied in the breeding, propagation, and conservation of the European chestnut. This includes traditional genetic transformation, genome editing techniques such as CRISPR/Cas9, in vitro culture, cryopreservation, and omics technologies—all integrated to enhance disease resistance (Figure 2).

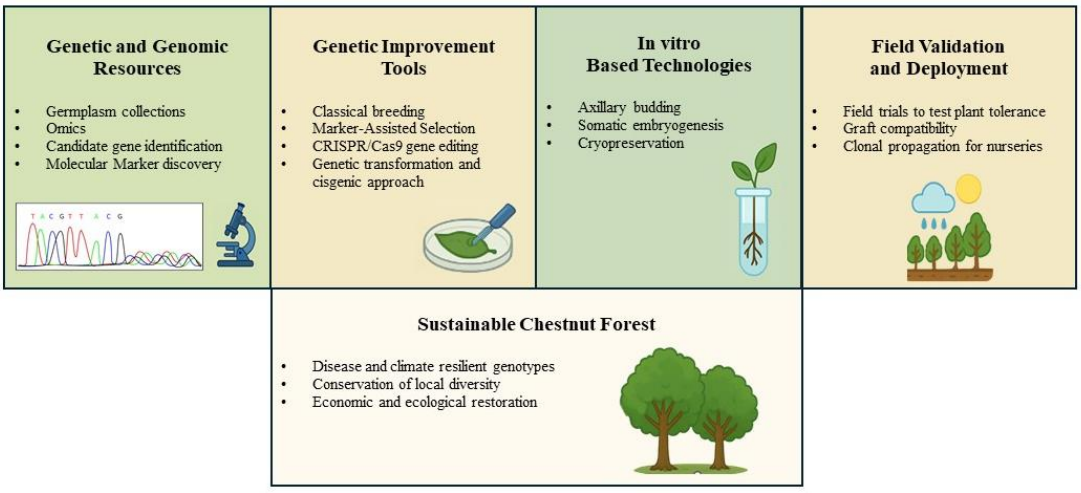

Figure 2. Schematic representation of the "lab-to-field" pipeline illustrating key strategies for developing improved European chestnut genotypes with enhanced resistance to pathogens, pests, and climate change. The

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diagram highlights integrative approaches, including molecular breeding, genomic selection, gene editing, phenotypic screening, and micropropagation.

2.1. Classical and Assisted Genetic Improvement

2.1.1. Controlled Hybridization

Interspecific hybridization has long underpinned the European chestnut breeding in Europe, particularly for enhancing resistance to ink disease and chestnut blight, but also for addressing other critical challenges in chestnut cultivation, such as timber production, nut quality, and grafting compatibility [38–42]. As mentioned above, beginning in the early 20th century, crosses between *C. sativa* and Asian species (*C. crenata* and *C. mollissima*) have provided the basis for breeding efforts aimed at introgressing desirable traits [43–47].

While *C. crenata* shows higher resistance to diseases, its poor climatic adaptation to early frost and drought conditions, undesirable phenotypic traits, and graft incompatibility with *C. sativa* cultivars have limited its direct use for nut or timber production [40,46,48]. Nonetheless, *C. crenata* has been the main source of ink disease resistance used in Europe, and several successful hybrid clones were derived from multiple breeding programs in France, Spain, Portugal, and Italy. These hybrids were commercialized for nut and timber production, as well as for use as rootstocks [48–51].

By contrast, *C. mollissima* has been particularly valued as a donor of resistance to chestnut blight and, to a lesser extent, for favourable nut traits such as ease of peeling and larger seed size. Although its direct adaptation to European environments is also limited, the incorporation of *C. mollissima* germplasm has complemented *C. crenata* in generating more balanced hybrids, combining tolerance to both pathogens with improved nut quality [45,46]. Several clones from Spanish breeding programs (111-1, 7521, 2671, and 1483) are widely used as rootstocks for their high tolerance to ink disease and high rootstock compatibility with fruit varieties [52]. In Portugal, more than fifty genotypes resistant to ink disease were selected during the first decade of the 2000s [53,54]. Following these efforts, the genetic improvement program initiated at Instituto Nacional de Investigação Agrária e Veterinária (INIAV) in 2006 placed particular emphasis on resistance to ink disease, given its continued negative impact on national orchards. This initiative also sought to modernize the plant material used in new plantations, particularly rootstocks, to ensure better adaptation to current climatic conditions, since most varieties then available still derived from breeding programs of the previous century [49,55,56]. Within this program, seven hybrid chestnut genotypes with enhanced resistance to ink disease were developed through controlled crosses (*C. sativa* × *C. crenata*/*mollissima*). The selection of these genotypes was based on inoculation trials conducted under controlled conditions on the progenies obtained from the crosses, allowing a rigorous characterization of their responses to *P. cinnamomi* infection. Disease severity was assessed using standardized symptom scales and mortality indices. Additionally, pathogenicity tests following Koch's postulates were conducted, ensuring reliable identification of the pathogen and confirming its causal association with the observed symptoms. The robustness of these tests was reinforced through inoculations on biological replicates of each genotype, obtained via micropropagation. The results revealed marked differences among genotypes, with some exhibiting significantly attenuated symptoms, reduced root necrosis, limited lesion progression to the collar, and higher survival rates [57]. Three of these genotypes are now registered in the Portuguese National Register of Fruit Varieties, including SC1202 (Variety RIVERA), which currently has an application under review for Community Plant Variety protection at the CPVO (Community Plant Variety Office). The propagation of these genotypes was achieved through micropropagation, enabling large-scale clonal production critical for validating *P. cinnamomi* inoculation trials, establishing field trials, conducting graft compatibility studies, transferring technology to nurseries, and conducting more fundamental studies like the identification of candidate genes of resistance. In parallel, the Spanish public company TRAGSA selected and officially registered seven new hybrid clones (denominated C003, C004, C042, C053, P011, P042, and P043) with resistance to ink disease in the Spanish National Catalogue of Basic Plant Materials.

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Other examples in France and Italy include Euro-Asian cultivars such as 'Bouche de Bétizac' and 'Primato', rootstocks such as 'Maridonne' and 'Marlhac', and rootstocks used directly as fruit producers 'Marigoule' and 'Maraval', which combine disease resistance with improved agronomic and adaptive traits [58–60]. More recently, some of these cultivars have also demonstrated resistance to the chestnut gall wasp [59,61,62]. Parallel breeding efforts have also been conducted outside Europe. In North America, *C. mollissima* and *C. crenata* have been used as donors of blight resistance in backcross programs with the American chestnut (*C. dentata*), with the aim of restoring timber chestnuts [63]. In Japan, programs have emphasized resistance to gall wasp together with nut traits such as ease of peeling [64].

Despite the success of these breeding programs, challenges remain in achieving optimal adaptation of hybrids to European climatic conditions. Over recent times, the intensification of drought and heat, acting synergistically with ink disease, has exacerbated damage in southern European chestnut stands [3,65]. Under these conditions, hybrid clones derived from *C. sativa* x *C. crenata* and *C. mollissima* have shown poor tolerance to these climate stressors [66–68]. In Spain, a breeding initiative was launched with the aim of developing trees tolerant to multiple stresses associated with global change [69–71]. Recently, two newly developed *C. sativa* clones have been reported to combine resistance to *P. cinnamomi* with tolerance to drought and heat, and they are currently undergoing registration at the Spanish Plant Variety Office [72].

Overall, progress in classical hybridization demonstrates the potential of interspecific breeding to provide genetic solutions for major chestnut diseases. However, the interaction of biotic and abiotic stresses under climate change highlights the need for integrated new breeding strategies. Future advances are expected to result from combining controlled hybridization with genome-assisted selection tools to accelerate the development of resilient *C. sativa* genotypes adapted to Mediterranean environments.

2.1.2. Perspective for Marker-Assisted Selection (MAS)

Advancements in molecular breeding must be integrated into chestnut breeding programs to support the increasing demand for elite chestnut genotypes. Thus, the application of marker-assisted selection (MAS) will be instrumental in accelerating the introgression of disease resistance and other beneficial traits such as environmental adaptability and agronomic performance. A foundational step was the development and deployment of molecular markers capable of identifying traits of interest and serving as tools for MAS programs. In *C. sativa*, genomic simple sequence repeats (gSSRs) were first developed approximately 20 years ago [73,74]. Since then, numerous studies have been conducted using these markers, and in perspective, they have provided the basis for MAS programs. Early applications of gSSRs demonstrated robust genotyping capacity, enabling accurate varietal discrimination [75–78]. For example, Pereira-Lorenzo et al. (2017) [79] used 24 highly polymorphic SSR markers to evaluate 271 accessions corresponding to 118 European cultivars, establishing a comprehensive European reference database for the identification and characterization of chestnut varieties.

In the Iberian Peninsula and surrounding regions, diversification and conservation of chestnut cultivars have also been explored using molecular tools. Pereira-Lorenzo et al. (2011) [80] performed a large-scale SSR-based survey on 593 grafted chestnut trees from Iberian Peninsula, and the Azores, and the Canary Islands, including more than 300-year-old individuals. They identified 356 distinct genotypes and reported a clonality rate of 33%, reflecting the widespread use of grafting. Despite clonal propagation, high genetic variability was maintained through hybridization and mutation, which emerged as the main driver of diversification. Ten cultivar groups were defined, exhibiting strong geographic structuring and evidence of long-distance dissemination to Atlantic islands, underscoring the role of human-mediated dispersal. The importance of molecular markers in distinguishing hybrids and detecting introgression from Asian species was highlighted by Pereira-Lorenzo et al. (2010) [81]. More recently, instant domestication has been highlighted to explain how traditional practices of selection and propagation rapidly shaped chestnut diversity [82]. The

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introduction of new sequencing techniques enabled the transformation of Single Nucleotide Polymorphisms (SNPs) into competitive allele-specific PCR (KASP) markers, successfully evaluated for varietal discrimination [83].

Moreover, information for MAS programs has also been obtained using functional markers such as Expressed Sequence Tag – Simple Sequence Repeats (EST-SSRs), which target expressed genes and therefore provide opportunities to directly associate genetic variation with adaptive traits. Their potential lies in supporting early selection for stress tolerance and disease resistance, complementing gSSR markers. Applications of EST-SSRs have included both the characterization of cultivated germplasm and of wild populations to evaluate the genetic and adaptive potential of chestnut in Europe [76,84]. Their potential has been further demonstrated through studies that revealed associations with water stress responses and tolerance to *P. cinnamomi*, supporting their use in the identification of drought-tolerant and disease-resistant individuals within breeding populations [26,69].

Population-level studies have further strengthened the case for functional markers. Studies using gSSRs revealed distinct gene pools across coppice and wild populations, shaped by geography and environmental gradients [85,86]. More recent work based on EST-SSRs expanded this approach, identifying loci potentially involved in stress responses. Castellana et al. [87], using the same EST-SSR markers to study variation across European chestnut populations, reported a potential association between the FIR059 allele and climatic variables, suggesting a role in abiotic stress adaptation. Similarly, Dorado et al. [70] used molecular markers associated with heat stress (VIT099 and POR016) to assess tolerance within and between populations, highlighting loci under positive selection. These markers stand out as promising candidates for early selection of heat-tolerant *C. sativa* individuals.

The availability of multilocus marker sets (SSRs, SNPs, double digest restriction-site associated DNA sequencing; ddRAD-seq) has also enabled the construction of high-density genetic linkage maps, which, combined with functional annotation of quantitative trait loci (QTL) intervals, provide a molecular basis for MAS and a platform for future genomic selection efforts. The first genetic linkage map for *C. sativa* [88], based on Random Amplified Polymorphic DNA (RAPD), Inter-Simple Sequence Repeat (ISSR), and isozyme markers, was constructed using the two-way pseudo-testcross strategy. This map facilitated the identification of QTLs associated with adaptive traits such as growth and water-use efficiency [89]. Comparative genetic and QTL mapping between *Quercus robur* L. and *C. sativa* identified homologous genomic regions, allowing putative candidate genes for bud burst to be inferred from colocations of EST-derived markers and QTLs [90].

Further refinement of genetic mapping has been achieved through bin mapping approaches, which cluster markers into cosegregating bins to improve map resolution and reduce redundancy, facilitating high-throughput genotyping and QTL discovery [91,92]. These genomic resources are now actively translated into MAS pipelines in *C. sativa* breeding programs. For example, breeders can pyramid resistance alleles for ink disease and gall wasp across generations using flanking SSR or SNP markers, while markers linked to heat tolerance allow early selection of individuals better adapted to increased temperatures. These approaches have already accelerated cultivar development, demonstrating how genomics-guided MAS complements classical breeding.

2.2. Molecular and Genomic Approaches

Extensive research aimed at understanding the genetic basis of resistance and susceptibility to chestnut's main biotic stresses has been driven by the urgent need to safeguard chestnut forests and orchard health. Advances in genetics, cell and molecular biology, and bioinformatics -complemented by insights from histopathology and physiology - have collectively built a robust foundation of knowledge, largely enabled by high-throughput sequencing technologies. Over the past two decades, genomic data generated by numerous research programs has rapidly grown, providing valuable resources for the development of effective and timely control and management strategies. Building on this foundation, the following section integrates and summarizes current knowledge from

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transcriptomic, proteomic, and metabolomic analyses, QTL mapping, molecular marker development, and whole-genome sequencing. It offers a comprehensive overview of the molecular and genomic underpinnings of chestnut defense against *P. cinnamomi, C. parasitica,* and *D. kuriphilus,* with a particular focus on European chestnut and its hybrids. The section also highlights candidate genes and pathways that may inform future resistance breeding and genetic engineering efforts, while outlining the genetic architecture that shapes chestnut responses to these biotic stresses.

2.2.1. Molecular Mechanisms of Castanea Defence Against *Phytophthora cinnamomi*

Molecular studies have revealed that resistance and tolerance to *P. cinnamomi* in *C. sativa* and its hybrids with Asian chestnuts involve a complex interplay of constitutive and inducible defense mechanisms, including gene expression regulation, biochemical barriers, and cellular responses [93–96].

A fundamental aspect of plant immunity is the presence of pre-formed, constitutive defenses that provide the first barrier against pathogen invasion. In the resistant *C. crenata*, high basal expression of defense-related genes such as those encoding receptor-like kinases (RLKs) and antifungal proteins (Cast_Gnk2-like) may fortify the root environment, rendering it less hospitable to pathogen colonization. By contrast, the susceptible *C. sativa* typically exhibits lower constitutive expression levels of those genes, predisposing it to a rapid initial invasion by *P. cinnamomi* [95]. The absence or low expression of pattern recognition receptors (PRRs), including BAK1 orthologs, in *C. sativa* may compromise its ability to perceive pathogen-associated molecular patterns (PAMPs) early during infection, thereby delaying the activation of downstream immune responses [93]. The weak pre-formed defence seems to contribute to the susceptibility of *C. sativa*.

A recurrent theme in the response of *Castanea* spp. to *P. cinnamomi* is the critical importance of timing. Resistant *C. crenata* exhibits an early activation of cellular defenses (within 0.5–2 h post-inoculation), which effectively curtails pathogen colonization [97]. These early cellular responses appear to reflect constitutive rather than induced gene expression, as evidenced by previously reported high expression levels of resistance-related genes in non-inoculated plants [95] and the limited induction of only a single gene 2 h post-inoculation [93]. By contrast, *C. sativa* demonstrates an early but transient activation of some defense components, followed by a marked decline in gene expression at 48–72 h post-inoculation. Notably, at the same time points, genes encoding for elicitins/elicitin-like (oomycete PAMPs) and necrosis-inducing like proteins (NLPs) were more expressed by *P. cinnamomi* in *C. sativa* compared to *C. crenata* [93]. This temporal mismatch not only allows *P. cinnamomi* to bypass the initial plant defenses but also creates a window during which pathogen effectors can further suppress host immune responses, promoting disease progression. Consequently, the continuum and sustainability of the defense response in resistant genotypes are critical determinants of successful resistance, while the inability of *C. sativa* to maintain these defenses results in elevated susceptibility.

The rapid and robust response of *C. crenata* includes the upregulation of genes encoding pathogenesis-related (PR) and antifungal proteins. In contrast, susceptible *C. sativa* displays delayed and weaker expression of these genes, resulting in more severe symptoms and higher mortality rates [94,95]. Santos et al. [95] conducted an in-depth analysis of gene expression responses in resistant (*C. crenata*), susceptible (*C. sativa*), and interspecific hybrids inoculated with *P. cinnamomi* using digital PCR. Among the eight candidate genes tested, clear genotype-dependent differences were observed. Remarkably, *Cast_Gnk2-like* exhibited not only a high constitutive expression level but also a rapid and strong activation in *C. crenata* following *P. cinnamomi* inoculation, whereas *C. sativa* showed low constitutive and induced levels of expression. Therefore, *Cast_Gnk2-like* emerged as a potential discriminator of resistance.

Cast_Gnk2-like shares homology with the ginkbilobin-2 (*Gnk2*) gene from *Ginkgo biloba* seeds. Gnk2 is a cysteine-rich repeat secreted protein harbouring a DUF26 domain with antifungal activity [98]. Gao et al. [99] reported that *Gnk2* is associated with the induction of Programmed Cell Death (PCD). Miyakawa et al. [100] suggest that *Gnk2* acts as a lectin and showed that it binds with high

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affinity to D-mannose, which may underlie its inhibitory effect on pathogen growth. As this carbohydrate is present in the cell wall of *P. cinnamomi*, some authors of this review hypothesized that the protein encoded by *Cast_Gnk2-like* could have a direct effect on the pathogen [101]. This hypothesis led to a series of functional validation studies within the genetic improvement program for *C. sativa* and other Fagaceae [102–104].

Comparative transcriptomic studies between resistant and susceptible *Castanea* species have revealed that resistant genotypes, such as *C. crenata*, upregulate a broader array of genes involved in pathogen perception, signal transduction, transcription factor activation, and the biosynthesis of defense metabolites [93,94]. In contrast, the transcriptome of *C. sativa* exposed to *P. cinnamomi* is characterized by a more limited set of differentially expressed genes, with lower overall expression levels of key defense markers. Proteomic analyses further corroborate these findings, indicating that proteins involved in salicylic acid (SA) signaling, reactive oxygen species (ROS) metabolism, and cell wall reinforcement are less abundant in infected *C. sativa* tissues [105].

Plant growth regulators (PGRs) play a crucial role in modulating immune responses against pathogens. Induction of SA-mediated responses results in the activation of PR proteins, cell wall reinforcement, and hypersensitive response (HR), which are key for resistance against hemibiotrophic pathogens [106]. Moreover, the interplay between SA and other hormones such as jasmonic acid (JA), abscisic acid (ABA), and ethylene (ET) is critical for fine-tuning the immune response. ABA often antagonizes SA-mediated responses.

A recent report, using physiological and biochemical methods, found that the response to *P. cinnamomi* in a resistant *C. sativa* x *C. crenata* genotype was characterized by early and strong SA signaling in roots, antagonism of ABA, stable primary and secondary metabolism, and transient oxidative stress with recovery [96]. On the other hand, the susceptible *C. sativa* response involves delayed and weak JA signaling, lack of SA induction, high ABA accumulation in leaves, impaired carbohydrate and secondary metabolism, and fluctuating oxidative stress that is not effectively countered (insufficient antioxidant response). Indeed, Santos et al. [95] suggested that SA signaling may be activated more rapidly in resistant genotypes than in susceptible ones, based on the expression patterns of *Cast_WRKY31* and Myb-related protein 4 (*Cast_Myb4*), transcription factors related to SAmediated responses.

Expression profiling of susceptibility genes (S genes) during *P. cinnamomi* infection provided further evidence supporting the delayed activation of SA-mediated defenses in the European chestnut [107]. In *C. sativa,* the S-genes *powdery mildew resistance 4* (*pmr4*, encoding a callose synthase) and *downy mildew resistance 6* (*dmr6*, repressor of the SA pathway) show early upregulation upon infection. This upregulation has been correlated with the suppression of SA-dependent defenses, thereby enhancing the susceptibility of the host. In contrast, *C. crenata* does not exhibit significant upregulation of these genes.

The allene oxide synthase gene from *C. crenata* (*CcAOS*), an ortholog of a key enzyme in the JA pathway, was identified from 2015 transcriptome data [94] after it was found to be significantly induced in inoculated *C. crenata* compared to *C. sativa*. The functional relevance of *CcAOS* in plant defense against *P. cinnamomi* was demonstrated by gene functional analysis, using genetic transformation to overexpress *CcAOS* in the susceptible *Arabidopsis* ecotype Ler-0, which resulted in delayed pathogen progression and enhanced tolerance [108].

Another essential mechanism of plant defense is the strengthening of the cell wall through the accumulation of phenolic compounds, which create physical barriers to pathogen cellular ingress and progression. Transcriptomic data indicate that the expression of genes linked to cell wall reinforcement such as those related to lignin biosynthesis and structural proteins is lower in *C. sativa* than in *C. crenata* [93]. This includes genes encoding cell wall-modifying enzymes such as Pectinesterase 2 (Cast_PE-2) and the TF (Cast_Myb4) [95]. These genes are upregulated promptly in resistant genotypes (*C. crenata* and *C. sativa* x *C. crenata*) after pathogen detection, which could lead to rapid cell wall thickening and reinforcement. Studies at the cellular level reveal these temporal

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differences, showing the accumulation of phenolic compounds in *C. crenata* and *C. sativa* cell walls at 30 min and 72 h after infection, respectively [97].

C. sativa can accumulate callose around intracellular hyphae as early as 24h after inoculation [97]. However, this cellular response, possibly mediated by S-gene *pmr4* [107], does not constitute an effective resistance defense. The inefficient and tardy activation of structural defenses allows *P. cinnamomi* to advance colonization and eventually cause widespread tissue necrosis. This is supported by the area of *P. cinnamomi* infection [97] and the quantity of pathogen DNA present in host tissues [107], which are significantly higher in *C. sativa* compared to *C. crenata.* Moreover, the lack of sustained activation of enzymes responsible for the cross-linking of cell wall components further exacerbates the vulnerability of *C. sativa* to pathogen invasion [93].

The rapid production of ROS is one of the hallmark responses of plants to pathogen attack, serving both as a direct antimicrobial agent and as a secondary signal to activate further defense responses. In resistant *C. crenata*, transcriptomics suggests a pronounced burst of ROS in infected tissues that may initiate HR, a rapid localized pathogen-induced cell death, thereby limiting pathogen spread [93,97]. While initial ROS generation may occur in *C. sativa* as well, this response may not be sustained; the transcription of genes coding for enzymes such as respiratory burst oxidase homolog protein B (*RBOHB*) is often transient, and the subsequent detoxification by antioxidative enzymes may further diminish the antimicrobial effects. Additionally, negative regulators of programmed cell death, such as BON1-associated protein 2-like (BAP2-like), are upregulated in *C. sativa*, possibly contributing to suppressing HR-like cell death and allowing *P. cinnamomi* to shift from biotrophic to necrotrophic lifestyle. The failure to maintain a robust ROS-mediated defense, coupled with premature suppression of HR, could be a key factor underlying the inability of *C. sativa* to restrict pathogen proliferation.

Dorado et al. [8] studied *C. sativa* defense against *P. cinnamomi* in warming scenarios and found that it depends on both morphological resilience (growth, root biomass) and the ability to accumulate specific phenolic compounds with antioxidant and antimicrobial properties: quercetin 3-O-glucuronide, 3-feruloylquinic acid, gallic acid ethyl ester, and ellagic acid. Briefly, plants previously exposed to moderate warming were more resilient to the pathogen, while those under normal or heat wave conditions were more susceptible. On the other hand, surviving plants after infection were characterized by increased levels of the four phenolic metabolites, which are central to the adaptive response to the combined challenges of heat and pathogen attack.

The Ubiquitin/26S proteasome system is integral to the dynamic regulation of plant immune responses through the targeted degradation of key proteins involved in hormone signaling and plant defense [109]. Some components of the system may also inhibit pathogen effectors by triggering PAMP and ETI responses [109]. Transcriptomics shows that *C. crenata* exhibits a high differential expression of *26S proteasome regulatory subunit 4 homolog A* (together with several proteases) after inoculation with *P. cinnamomi*, when compared to *C. sativa* [93]. The apparent absence of regulation of the Ubiquitin/26S proteasome system in *C. sativa* conceives the persistence of negative regulators that dampen defense responses. Although the precise components of this regulatory network in chestnut remain to be elucidated, current evidence underscores the importance of post-translational modifications in fine-tuning the balance between resistance and susceptibility during *P. cinnamomi* infection.

2.2.1.1. Genetic Basis of *P. cinnamomi* Resistance: Marker Development and Quantitative Trait Loci (QTL) Mapping

Building on the availability of genomic resources, including transcriptomic data [94], Santos et al. [110] developed 43 novel EST-SSR markers. These markers, designed from differentially expressed genes associated with host responses to infection, showed high amplification success and interspecific transferability across four *Castanea* species. The average expected heterozygosity (0.61) was higher than previously reported for chestnut EST-SSRs, confirming their utility for genetic studies.

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An important advance for *C. sativa* breeding programs was the construction of the first interspecific genetic linkage map for *C. sativa* × *C. crenata* [111]. This map was constructed using 452 SSRs and SNPs and spans 498.9 centimorgans (cM). It enabled the detection of QTLs for ink disease resistance on linkage groups E and K, which overlap with QTLs from American x Chinese chestnut populations [112], suggesting conserved *P. cinnamomi* defense mechanisms across chestnut species. Additionally, QTLs on linkage group E co-localized with defense-related genes, including those putatively encoding PR proteins (NDR1/HIN1-Like protein 3), phospholipid transporters, transcriptional regulators (RNA polymerase II-associated factor 1; PAF1 homolog), and epigenetic modulators (Zinc-finger PHD-type)[111].

Genetic linkage maps and the identification of QTLs associated with ink disease resistance have revealed the polygenic nature of this trait in chestnut. These studies not only provided evidence of the genetic architecture underlying ink disease resistance in chestnut but also delivered molecular markers with strong potential for MAS. Table 1 summarizes genomic resources and key insights into the responses of susceptible and resistant chestnuts to *P. cinnamomi* infection.

2.2.2. Molecular Mechanisms of Castanea Defense Against *Cryphonectria parasitica*

Chestnut blight poses a significant threat to chestnut species worldwide. While American chestnut is highly susceptible, the European chestnut displays relatively lower susceptibility and, in some cases, tolerance. Among the Asian species, the Chinese chestnut is considered more resistant to blight than the Japanese chestnut, which is attributed to a combination of rapid immune response activation, efficient pathogen recognition, and robust structural defenses [113].

2.2.2.1. Castanea sativa: Partial Tolerance or Susceptibility to Blight

C. sativa can be susceptible to *C. parasitica*, particularly to virulent strains that cause extensive necrosis and canker formation. The fungus typically penetrates into the host tissue through wounds or bark cracks, often facilitated by environmental stressors like drought or mechanical damage. Once inside, the pathogen forms mycelial fans that exert physical pressure and enzymatically degrade host tissues using laccases, cellulases, and cutinases. Additionally, the pathogen produces phytotoxic secondary metabolites, including skyrin, skyrinol, rugulosin, diaportin, and nitrogen-containing compounds, which cause rapid necrosis in host tissues. Apical shoots and leaves are particularly sensitive to these toxins, with mortality observed within 8 days [114]. The host tree attempts to respond by lignifying cell walls to reinforce structural barriers and forming wound periderm, a protective layer to isolate infected tissue. However, these defenses are consistently suppressed by the advancing fungal mycelium. Instances of limited recovery of infected trees have been documented under certain environmental conditions or in specific genotypes; these occurrences are best interpreted as examples of tolerance rather than resistance [115]. Tolerance allows trees to survive and maintain some functionality despite the presence of the pathogen, but it does not preclude initial infection or the subsequent establishment of cankers.

Table 1. Biotechnological tools to study chestnut responses to *Phytophthora cinnamomi*, focused on *Castanea sativa* (susceptible) and *Castanea crenata* (resistant).

Methodology	Species	Main Findings	References
Comparative transcriptomics	*C. crenata* *C. sativa*	*C. crenata* upregulates genes for pathogen perception, signaling, transcription factors, and defense metabolites. *C. sativa* shows limited and transient expression	[93,94]
Molecular marker development	*C. sativa* *C. crenata*	43 EST-SSR markers identified from DEGs associated with host responses to infection	[110] supported by results in [94]
Genetic mapping	*C. sativa x C. crenata*	Interspecific linkage map enabled detection of QTLs for pathogen resistance on linkage groups E and K, co-localizing with defense-related genes	[111] supported by results in [110]
Gene expression profiling	*C. crenata* *C. sativa*	*C. crenata* shows high basal and induced expression of PR genes (e.g., *RLKs*, *Cast_Gnk2-like*), enabling early	[93,95] in accordance with results in [97]

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	C. sativa x C. crenata	defense activation. *C. sativa* has lower expression, allowing rapid pathogen colonization	
Functional gene validation	*C. sativa* *C. dentata* *Quercus ilex* *Quercus suber* *Arabidopsis thaliana*	*Cast_Gnk2-like* relevant in *Castanea* and *Quercus* defense; *CcAOS* enhances tolerance in *A. thaliana* Ler-0	[102–104,108] corroborated by results in [95]
Proteomics	*C. sativa*	*C. sativa* upon infection shows downregulation of proteins involved in SA signaling	[105]in accordance with results in [93,94]
Histopathology and cellular studies	*C. sativa* *C. crenata*	*C. crenata* responds more efficiently than *C. sativa;* pathogen's growth is restricted by early activation of callose deposition, HR-like cell death, cell wall thickening and accumulation of phenolic-like compounds.	[97] in accordance with results in [93,107]
Susceptibility gene expression analysis	*C. sativa* *C. crenata*	*C. sativa* upregulates *pmr4* and *dmr6* early in the infection, putatively contributing to suppressing SA defenses; putative callose accumulation via *pmr4* is not sufficient to restrict pathogen growth	[107] in accordance with results in [97]
Metabolite analysis	*C. sativa*	Moderate warming enhances *C. sativa* resilience to pathogen. Surviving plants accumulate key phenolics (e.g., quercetin 3-O-glucuronide, ellagic acid), contributing to defense	[8]
Physiological and biochemical assays	*C. sativa* *C. sativa x C. crenata*	*C. sativa* × *C. crenata* show early SA signaling, ABA antagonism, and oxidative stress recovery. *C. sativa* shows delayed JA signaling, high ABA, impaired metabolism, and weak antioxidant response	[96] in accordance with results in [95]

ABA: Abscisic acid; *CcAOS*: Allene Oxide Synthase; DEGs: Differentially expressed genes; *dmr6*: Downy Mildew Resistant 6; EST-SSR: Expressed Sequence Tag – Simple Sequence Repeats; HR: Hypersensitive response; JA: Jasmonic acid; *pmr4*: Powdery Mildew Resistant 4; PR: Pathogenesis-related; QTL: Quantitative Trait Loci; RLKs: Receptor-like kinases; SA: Salicylic acid.

At the physiological and biochemical level, responses of *C. sativa* to *C. parasitica* include [116]:

- Lowering of photosynthetic pigments and augmentation of antioxidant enzyme activities (Ascorbate peroxidase (APX), Guaiacol peroxidase (POD), and Superoxide dismutase (SOD)).
- Accumulation of the stress markers proline (an amino acid that in stress conditions acts as an osmolyte, stabilizes proteins, and neutralizes ROS) and malondialdehyde (a marker of lipid peroxidation caused by oxidative stress levels in infected tissues).

Despite *C. sativa* susceptibility to *C. parasitica*, the presence of hypovirulent fungal strains and the use of biological control agents like *Cryphonectria hypovirus 1* (CHV1) have shown promise in mitigating disease severity in Europe [117]. These strains exhibit reduced growth and sporulation and cause superficial necrosis that may heal over time (with the development of calli), allowing the tree to compartmentalize the infection [118]. Also, hypovirulent strains produce lower levels of virulence enzymes like laccase [114]. In another set of experiments, hybrids between *C. sativa* and *C. crenata* were subjected to inoculation with both virulent and hypovirulent strains [119,120]. These studies confirmed that while the hybrids can showcase enhanced tolerance and, in some cases, genotype-dependent recovery, the pure *C. sativa* lines included in the trials remained susceptible to the pathogen, thereby underscoring the absence of innate immunity.

Chitinases are hydrolytic enzymes that break down chitin, a key component of fungal cell walls, and are considered crucial inducible defense proteins in plants [121]. Gene and protein expression studies in *C. sativa* showed systemic induction of chitinase (and β-1,3-glucanase) in response to infection, with higher activity observed in trees inoculated with hypovirulent strains [122,123]. Vannini et al. (1999) [124] purified four chitinases from *C. sativa* and found that three of them inhibit hyphal growth in vitro. Also, hypovirulent strains were more susceptible than virulent ones, suggesting that slower hyphal development in hypovirulent strains may be related to vulnerability

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to host chitinases. These findings imply that the hypovirulence-associated virus may enhance host recognition or defense responses. The endochitinase-like protein Ch3, isolated from *C. sativa* cotyledons, showed antifungal properties in vitro [125], and its corresponding gene was isolated for further validation as a resistance gene [126]. More details are given in section 2.4.1.

Pavese et al. (2021) [107] conducted gene expression profiling of *C. sativa* inoculated with *C. parasitica.* Their findings revealed that the upregulation of S genes such as *pmr4* and *dmr6* (more detailed in 2.2.1) may activate stress pathways that inadvertently facilitate the pathogen's progression by downregulating SA-mediated responses. Also, investigations into the expression of PR proteins, including chitinases and glucanases, demonstrated significant induction upon fungal infection.

SA is a signaling molecule largely associated with plant defense, particularly in activating pathogen resistance genes [127]. Biochemical studies in *C. sativa* show SA accumulation following inoculation with both virulent and hypovirulent strains of *C. parasitica*, with significantly higher levels in trees infected by hypovirulent strains, suggesting either enhanced host response or suppression by virulent strains [123]. This pattern mirrors findings in chitinase gene expression, where hypovirulence was associated with stronger host activation [123]. Transcriptomic analyses in *C. dentata* and *C. mollissima* also revealed increased SA-related gene expression in canker tissue, supporting its role in defense across species [128]. The literature identifies SA signaling activation as a multifaceted process that involves the induction of defense genes, cell death regulation, and antagonism/fine-tuning of JA-ET signaling. This activation is both a host defensive strategy and, in some cases, a target for pathogen manipulation [127,129].

Overall, *C. sativa* exhibits partial and often ineffective defense responses to *C. parasitica*. Defense suppression by the fungus may be a hallmark of *C. sativa*'s limited resistance compared to Asian chestnut species. The presence of hypovirulent strains offers some hope for disease mitigation, but the species remains vulnerable without external intervention or genetic improvement.

2.2.2.2. Castanea mollissima: Robust Genetic Resistance

C. mollissima has co-evolved with the pathogen in its native range, resulting in the development of genetically encoded resistance mechanisms. This species exhibits both constitutive and inducible defenses, including rapid wound response, cell wall lignification, and the activation of resistance genes upon infection. These traits are supported by genomic studies that have identified candidate genes associated with disease resistance, particularly in the sequenced genome of the cultivar 'Vanuxem', which has been widely used in restoration breeding programs in North America and Europe [130].

The reference genome of *C. mollissima* reveals a well-structured genetic architecture, with over 36000 gene models and extensive transcriptomic data. These resources have enabled the identification of selection signatures and resistance loci that differentiate *C. mollissima* from susceptible species like *C. dentata* and *C. sativa* [130]. Moreover, the species shows high genetic diversity across its natural populations, especially in regions like the Qinling-Daba Mountains, which are considered reservoirs of resistance genes [131].

Additionally, the complete chloroplast genome of wild *C. mollissima* has been sequenced, providing further insights into its evolutionary adaptations. This genome includes 131 genes, many of which are involved in stress responses and metabolic regulation. Phylogenetic analyses confirm its close relationship with other resistant Fagaceae species, reinforcing its role as a genetic donor in breeding programs [132].

2.2.2.3. Breeding and Genomic Efforts

The stark contrast between *C. sativa* and *C. mollissima* has driven interspecific hybridization efforts, aiming to introgress resistance traits from the latter into the former (described in 2.1.1).

However, blight is most severe on the American chestnut. A review by Fernandes et al. [3] outlined decades of efforts to combat chestnut blight in the US, recurring principally to crosses between *C. dentata* and the cornerstone of chestnut blight resistance, *C. mollissima*. Hybridization

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efforts began in the 1920s; although first-generation hybrids showed strong resistance, they lacked the tall growth typical of *C. dentata*. To recover these growth traits, backcrossing to *C. dentata* was pursued, based on the idea that a few major alleles from *C. mollissima* conferred resistance to *C. parasitica* and *P. cinnamomi* [45,133]. Advanced backcross hybrids resembled *C. dentata* and had improved resistance, but molecular studies revealed a tradeoff between blight resistance and *C. dentata* ancestry. This suggests that blight resistance is a multigenic and quantitative trait, as supported by genetic mapping that identified resistance loci on all 12 chromosomes [63,134].

Westbrook et al. [135] recently provided a comprehensive overview of US breeding programs aimed at developing self-sustaining populations of American chestnut with resistance or tolerance to both *C. parasitica* and *P. cinnamomi*. The authors employed an integrative approach combining genomics, transcriptomics, and statistical modeling, establishing a robust framework to accelerate restoration efforts through both conventional breeding and biotechnological strategies. Recognizing the potential of gene editing as a precise and rapid tool, they identified candidate genes associated with resistance and susceptibility, many of which co-localize with previously identified QTLs for blight resistance. To support this, new whole-genome sequences of *C. dentata* 'Ellis' and *C. mollissima* 'Mahogany' were generated and used in genome-wide association studies (GWAS). Among the findings, four genes, including a chitinase, were highlighted as potential resistance factors, while 26 genes were proposed as susceptibility candidates.

Transgenics represents a complementary strategy to the labor-intensive process of backcross breeding. Transgenic *C. dentata* expressing the wheat *OxO* gene have shown promising results and are currently undergoing regulatory review and field trials [136,137]. The introduced oxalate oxidase enzyme enables the degradation of oxalic acid secreted by *C. parasitica*, producing carbon dioxide and hydrogen peroxide. This reaction mitigates the damaging effects of the pathogen by reducing the severity of fungal-induced lesions [138]. The US Department of Agriculture Animal and Plant Health Inspection Service (USDA-APHIS) completed a draft review concluding that the transgenic American chestnut is unlikely to pose a plant pest risk [139]. Other US Federal agencies (Environmental Protection Agency; Food and Drug Administration) still need to complete their reviews. Nonetheless, the potential approval of this transgenic tree will represent a major milestone in the application of transgenesis for ecological conservation.

2.2.2.4. Transcriptomic Insights into Chestnut Blight Resistance

Barakat et al. [140] used high-throughput pyrosequencing for transcriptome comparison between blight-susceptible American chestnut and blight-resistant Chinese chestnut in response to infection by *C. parasitica*. Researchers sequenced RNA from both healthy and infected stem tissues, generating over a million reads and assembling tens of thousands of unigenes for each species.

Key findings include: a) A large number of genes associated with stress and biotic stimulus responses were identified, with many showing differential expression between healthy and infected tissues with canker; b) Defense-related genes such as transcription factors (e.g., *WRKY, zinc finger, MYB*), enzymes (e.g., cinnamoyl-CoA reductase, alpha-dioxygenase), and transporters were more highly expressed in canker tissues; c) *C. mollissima* exhibited a broader and more robust transcriptional response to infection, consistent with its higher resistance; d) Functional annotation revealed similar overall gene function distributions between species, but with subtle differences in categories like transcription factor activity and stress response.

Sequentially, Barakat et al. [128] deepen the findings in Barakat et al. [140]. Transcriptome comparative analysis revealed that chestnut genes share greater similarity with other woody plants than with herbaceous species. The study identified hundreds of genes with differential transcript abundance between cankered and healthy stem tissues. Many of these genes are involved in key defense-related pathways, including cell wall biosynthesis, ROS production, hormone signaling (e.g., SA, JA, ET, ABA), HR, and PCD. HR and PCD are generally associated with immune responses to biotrophic organisms. However, studies have consistently shown that necrotrophic pathogens exploit the dead tissue generated by HR, thereby using the host's defense machinery to support their own

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life cycles [141]. Molecular studies further complicate the picture by demonstrating that HR and PCD are not universally coupled to resistance; genetic analyses have shown that HR and PCD components can be activated independently and that resistance against necrotrophic pathogens may sometimes be achieved with limited cell death [142].

Peroxidase activity may be involved in the host response to *C. parasitica*, with increased transcript levels observed in canker tissue of *C. mollissima* [128]. Peroxidases, namely the ones of Class III, are a group of secretory plant enzymes involved in the one-electron oxidation of various substrates, producing peroxides, and are strongly associated with induced pathogen defense mechanisms [121,143]. Beyond their defensive role, they also contribute to auxin metabolism, cell wall reinforcement, lignin and suberin synthesis, and the regulation of reactive oxygen and nitrogen species [144]. In *Castanea* species, four distinct peroxidases have been identified in stem and leaf tissues [145], though their enzymatic properties remain poorly characterized. Although the mentioned studies suggest that peroxidase activity may play a role in the host response to *C. parasitica*, conflicting findings have been reported: *Castanea pumila* Mill., a blight-susceptible species, showed higher peroxidase transcript abundance than the resistant *C. mollissima* [146], and infection was found to reduce peroxidase activity in both *C. dentata* and *C. mollissima*, with a smaller decrease in the latter [145]. These findings suggest that *C. parasitica* may suppress host peroxidase activity, particularly in susceptible species, but the precise role of peroxidases in chestnut blight resistance remains unclear due to the lack of follow-up studies.

The findings of Barakat et al. [128,140] provide valuable genomic resources and identify candidate genes and networks potentially responsible for host resistance to *C. parasitica*. Although the gene expression profiles activated in canker tissues by *C. parasitica* were largely similar between the two species, notable differences were observed: *C. dentata* exhibited increased activation of housekeeping genes, whereas *C. mollissima* showed a stronger induction of PR transcripts. The authors hypothesized that the difference in blight tolerance between *C. dentata* and *C. mollissima* may depend more on the speed of the host's response to infection than on the presence of specific defense-related genes.

To better understand the molecular basis of blight resistance in *C. mollissima*, Nie et al. [147] performed transcriptomic profiling of the resistant wild genotype 'HBY-1' at 0, 3, and 9 h post-inoculation with *C. parasitica*, after Illumina sequencing. The analysis revealed that 9 h post-infection is a critical time point for defense activation. Two hundred and eighty-three differentially expressed genes (DEGs) were identified and grouped into two major functional clusters: a) Metabolism-related pathways, including biosynthesis of secondary metabolites, phenylpropanoid biosynthesis, and photosynthesis. b) Defense-related pathways, such as plant-pathogen interaction and MAPK signal transduction. These clusters were interconnected through signaling systems involving phosphatidylinositol, phytohormones, and α-linolenic acid metabolism. Notably, genes involved in JA biosynthesis and metabolism were significantly upregulated, indicating full activation of the JA pathway early in the infection process. The study concludes that *C. mollissima* mounts a rapid and coordinated defense involving hormone signaling, pathogen recognition, and metabolic reprogramming.

2.2.2.5. Metabolomic Insights into Chestnut Blight Resistance

The metabolomic dynamics governing the interaction between *C. parasitica* and *Castanea* species remain largely unexplored. The review by Lovat and Donnelly [113] provides a comprehensive analysis of the mechanisms and metabolites involved in the interaction between chestnuts and *C. parasitica*.

In the chestnut cell walls, compounds related to the lignin barrier and wound periderm involved in blight defense have been identified. Tannins, a diverse group of polyphenolic compounds including phenolic acids, flavonoids, and sugars, play key roles in chestnut species by contributing to plant defense, antioxidant activity, and structural integrity [148]. Their interaction with the pathogen *C. parasitica* is multifaceted: while fungal growth may be enhanced by tannin-rich media

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due to tannase activity [149,150], species-specific tannin profiles influence disease resistance. Asian chestnuts like *C. mollissima* contain higher levels of vescalagin and castalagin, which may inhibit fungal growth more effectively than hamamelitannin, prevalent in the susceptible *C. dentata* and *C. sativa* [113]. Histological and hormonal studies suggest that esterase activity and JA signaling also modulate tannin availability and response to infection [151,152]. Moreover, tannins may inhibit fungal enzymes such as polygalacturonase, further complicating their role in pathogenesis [153]. Overall, tannins are central to the host–pathogen dynamics in chestnut blight, acting both as potential substrates and modulators of fungal virulence. A synthesis of the strategies and key findings for understanding chestnut responses to *C. parasitica* is presented in Table 2.

Table 2. Biotechnological tools to study chestnut's resistance to *Cryphonectria parasitica*, focused on *C. sativa* (tolerant/susceptible), *C. dentata* (susceptible), and *C. mollissima* (resistant).

Methodology	Species / Genotypes	Results / Findings	References
Physiological and biochemical responses	*C. sativa*	Reduced photosynthetic pigments; Increased APX, POD, SOD; Accumulation of proline and MDA	[116]
Biological control CHV1 hypovirus	*C. sativa*	Mitigation of disease severity via hypovirulent strains	[117]
Chitinase and β-1,3-glucanase expression	*C. sativa*	Systemic induction; Higher activity with hypovirulent strains; Antifungal activity of Ch3 protein	[122–125]
Susceptibility gene expression profiling	*C. sativa*	Upregulation of *pmr4* and *dmr6*; Suppression of SA-mediated responses	[107]
SA accumulation studies (metabolite and transcriptome analysis)	*C. sativa* *C. dentata* *C. mollissima*	Higher SA levels with hypovirulent strains; SA-related gene expression in canker tissue	[123,128]
Genomic and transcriptomic studies	*C. mollissima* 'Vanuxem'	Identification of resistance genes; Rapid wound response and cell wall lignification	[130]
Chloroplast genome sequencing	Wild *C. mollissima*	131 genes involved in stress responses and metabolic regulation	[132]
Genetic mapping and GWAS	*C. dentata* × *C. mollissima*	Resistance loci on all chromosomes; Candidate resistance and susceptibility genes identified	[63,134,135]
Transgenic *OxO* expression	*C. dentata*	Oxalate oxidase degrades oxalic acid from pathogen; field trials and regulatory review ongoing	[136,137]
Transcriptome comparison via pyrosequencing	*C. dentata* vs. *C. mollissima*	Differential expression of defense genes; Stronger defense response in *C. mollissima*	[128,140]
Transcriptomic profiling	Wild *C. mollissima* 'HBY-1'	283 DEGs in metabolism and defense pathways; Early JA pathway activation	[147]
Tannin profiling (metabolite analysis)	*C. mollissima* *C. dentata* *C. sativa*	Prevalence of hamamelitannin in *C. sativa* and *C. dentata*; Higher vescalagin and castalagin *in C. mollissima* - inhibition of fungal enzymes	[151–153] [113](and references within)

APX: Ascorbate peroxidase; ch3: Chitinase; CHV1: *Cryphonectria* hypovirus 1; DEGs: Differentially expressed genes; *dmr6*: Downy Mildew Resistant 6; GWAS: genome-wide association studies; JA: Jasmonic acid; MDA: Malondialdehyde; OxO: Oxalate oxidase; *pmr4*: Powdery Mildew Resistant 4; POD: Guaiacol peroxidase; SA: Salicylic acid; SOD: Superoxide dismutase.

2.2.3. Molecular Mechanisms of Castanea Defense Against *Dryocosmus kuriphilus*

The molecular interactions between the chestnut gall wasp and the European chestnut (and its hybrids) remain poorly investigated, particularly the genetic bases of host resistance and susceptibility. Compared with research on chestnut blight and ink disease, progress has been slower,

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likely due to the pest's later introduction in Europe, its initially underestimated ecological and economic impacts, and the early reliance on biological control. Most studies have focused on evaluating and implementing a biological control using the parasitoid wasp *Torymus sinensis* Kamijo, which is considered the most effective strategy for reducing forest infestations. Biological control has been deployed in several countries, including Japan, Korea, Italy, and the US, yet infestations continue to impact harvests and the cynipid continues to spread in Europe [21,59,154,155]. However, orchard-level containment may benefit from the use of genotypes carrying resistance or reduced susceptibility.

Identifying resistant genotypes is essential to understanding the chestnut-pest interactions for breeding programs. Resistance to *D. kuriphilus* has been documented in the hybrid Bouche de Bétizac (*C. sativa* x *C. crenata*). Early studies reported no infestations for 3 years [156], and this resistance was later confirmed after nearly a decade of observations [59]. Although the wasp can still lay eggs in the buds, larvae fail to develop beyond the first instar, most likely due to HR triggered by the hybrid. Dini et al. [157] demonstrated this by using diaminobenzidine (DAB) staining to detect in vivo H_2O_2 accumulation, an indicator of the stress-response glycoproteins germin and germin-like proteins (GLPs). GLPs, which have OxO activity, are associated with PCD and HR. Positive DAB staining was observed in Bouche de Bétizac buds but not in those of the susceptible cultivar Madonna (*C. sativa*), regardless of infestation state. Furthermore, strong expression of a putative GLP was detected in Bouche de Bétizac tissues during early budburst, reinforcing the presence of HR. This response was also proposed in preliminary transcriptomic studies; using Differential Display analysis between Bouche de Bétizac and the susceptible cultivar Marrone (*C. sativa*), Botta et al. [158] identified differentially expressed bands with sequences putatively encoding for resistance genes, mitogen-activated proteins, vesicle-associated membrane proteins, and 14-3-3 proteins.

The molecular basis of resistant and susceptible responses to *D. kuriphilus* infestations was investigated by Acquadro et al. [159] using transcriptomic analysis of buds (pools of several gall development stages) from Bouche de Bétizac and Madonna. The two assemblies contained 34,081 and 30,605 unigenes, respectively. For downstream analyses, the Bouche de Bétizac unigene set was functionally characterized, whereas the Madonna assembly was used mainly for RNA-seq data analysis. This work identified 1,444 putative resistance gene analogs (RGAs) and approximately 1,135 unigenes predicted as miRNA targets. Global transcriptome profiling revealed significant Gene Ontology enrichments, particularly for *response to stimulus* and *developmental processes* (e.g., post-embryonic development). Among the up-regulated genes, several were associated with attack recognition, with approximately 60 predicted to encode leucine-rich repeat (LRR) proteins; others were related to *transcriptional regulator activity*, including 6 APETALA2/Ethylene (AP2/ERF) and 16 WRKY (e.g., WRKY33). A putative homolog to RAV1 transcription factor was also upregulated; beyond its proposed role as a negative regulator of growth and development, Acquadro et al. (2020) [159] suggested that RAV1 regulation may also serve as a developmental adaptation to gall-induced stimuli. Additional examples of upregulated genes included protein regulators (e.g., Regulatory-Associated Protein of TOR 1b; RAPTOR1B), storage proteins (e.g., Late Embryogenesis Abundant protein D29; LEA D29), and more than 100 genes linked to *death* and *apoptosis* processes, including those involved in HR response [159]. These findings further support the HR-based mechanism previously suggested by Dini et al. [157]. In addition to clarifying molecular responses, the study produced valuable resources, including the first reference unigene catalog for the European chestnut, along with ˜7k SSR and 335k SNP/INDEL markers.

Another breakthrough in understanding the genetic resistance of Bouche de Bétizac came from high-density mapping in interspecific hybrids. A large-effect QTL, Rdk1, was mapped to linkage group K, explaining 67–69 % of the phenotypic variance [61]. Within this region, 26 candidate genes were identified, including two of particular interest - *metacaspase-1b* and a receptor of the Resistance to *Peronospora parasitica 13* locus (RPP13) subfamily. Both genes are known to be involved in HR.

In addition to Bouche de Bétizac, Sartor et al. [59] identified six other cultivars resistant to *D. kuriphilus*, including two *C. sativa* cultivars: the Italian 'Pugnenga' and the French 'Savoye'. This

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finding is particularly significant, as it suggests the possibility of transmitting resistant traits within the species. More recently, resistance was detected in natural populations of *C. sativa* in Greece [62]. A genome-wide association study provided the first genomic insights into this resistance, revealing a small region on pseudochromosome 3 (Chr3) associated with the high resistance observed in Greek provenances. This region harbors 12 candidate genes, including members of the *Cytochrome P450, UDP-glycosyltransferase,* and *Rac-like GTP-binding protein* families. Notably, 21 SNPs within this region were identified, offering promising markers for MAS in breeding programs.

Insights into resistant and susceptible responses of other chestnut species to *D. kuriphilus* can provide valuable guidance for European chestnut research and breeding programs. Zhu et al. [160] suggested that the peroxidase pathway may contribute to resistance in a partially resistant Chinese chestnut variety, and identified 4 transcription factors as potential players in this pathway (*CmbHLH130, CmWRKY31, CmNAC50,* and *CmPHL12*).

Although further research is needed to fully clarify the genetic basis of chestnut responses to gall wasp, current evidence consistently highlights oxidative stress signaling as a central mechanism. Several candidate resistance genes have already been identified, providing a strong foundation for future functional studies. Moreover, the recent publication of a high-quality reference genome for *D. kuriphilus* [161] opens new opportunities to investigate the pest's molecular weapons and the associated host gene expression changes during infestation. Such insights will enable a more comprehensive understanding of chestnut-gall wasp interactions and inform the development of integrated management strategies (beyond the ongoing biological control with *T. sinensis*) to mitigate the impacts of this invasive cynipid. Table 3 provides an overview of the strategies used to understand chestnut's resistance to chestnut gall wasp.

2.2.4. Whole Genome Sequencing

Whole genome sequencing (WGS) has become a cornerstone in advancing molecular knowledge of defense mechanisms in chestnut species, particularly in response to pathogens, pests, and climate change. By providing comprehensive insights into the genetic architecture of chestnut trees, WGS enables the identification of resistance-associated genes and regulatory elements that govern responses to biotic and abiotic stressors.

Genome assemblies differing in completeness are now available for the four chestnut species. For *C. sativa*, two assemblies have been published: one for the cultivar 'Marrone di Chiusa Pesio' using Oxford Nanopore and Illumina technologies [162] and another for the Anatolian cultivar 'Sarı Aşılama' [163]. The genome of *C. mollissima* has been sequenced for multiple cultivars, including 'Vanuxem', a donor of blight resistance in *C. dentata* restoration breeding [130]. For *C. crenata*, a chromosome-level genome assembly has revealed conserved chromosomal segments and a large repertoire of protein-coding genes, supporting its known resistance to diseases and pests [164].

These genomic resources facilitate comparative analyses that identify candidate genes under selection and enable MAS for resistance traits. WGS also supports landscape genomics approaches, mapping adaptive genetic variation across environmental gradients and predicting genomic offset under future climate scenarios [165]. Furthermore, genome-enabled breeding programs are incorporating adaptive diversity into backcross populations, ensuring that restored American chestnut trees are not only disease-resistant but also ecologically viable across diverse habitats [135].

Table 3. Biotechnological strategies to understand the European chestnut's resistance to *Dryocosmus kuriphilus*.

Methodology	Species / Genotypes	Results / Findings	References
Biological control *Torymus sinensis*	Various (wild and cultivated)	Effective in reducing infestations, but pest continues to spread	[21,154,155]
Phenotypic resistance screening	*C. sativa, C. crenata* and Euro japanese hybrids	7 resistant cultivars identified: *C. sativa* 'Pugnenga' & 'Savoye'; *C. crenata* 'Idae'; Hybrids 'BB', 'Marlhac', Maridonne', 'Vignols'	[59,156]
Histochemistry and	'BB' (R) vs. 'Madonna' (*C. sativa*, S)	Detection of H_2O_2 accumulation and strong GLP expression in R hybrid linked to HR	[157]

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gene expression			
RNA-seq transcriptome analysis	'BB' (R) vs. 'Madonna' (*C. sativa*, S)	1,444 RGAs, 1,135 miRNA targets; upregulation of *LRRs, WRKYs, AP2/ERFs, RAV1, LEA D29, RAPTOR1B*; HR-related genes	[159]
	C. mollissima 'Shuhe Wuyingli' (PR) vs. 'HongLi' (S)	Peroxidase pathway implicated; 4 TFs identified (CmbHLH130, *CmWRKY31, CmNAC50, CmPHL12*)	[160]
Genomic resources development	*C. sativa*	Reference unigene catalog; ~7k SSRs and 335k SNP/INDELs	[159]
QTL mapping	Interspecific hybrids 'BB' x 'Madonna'	Rdk1 locus explains 67–69% of resistance variance; candidate genes include *metacaspase-1b* and *RPP13 receptor*	[61]
GWAS	Greek *C. sativa* provenances (R)	Region on Chr3 with 12 candidate genes (*Cytochrome P450, UDP-GT, Rac-like GTPases*); 21 SNPs identified	[62]
Genome sequencing	*D. kuriphilus* (pathogen)	High-quality reference genome published; enables host-pest interaction studies	[161]

BB: 'Bouche de Bétizac'; GLP: germin-like protein; GWAS: genome-wide association study; HR: hypersensitive response; INDEL: insertion deletion; PR: partially resistant; QTL: quantitative trait loci; R: resistant; RGAs: resistance gene analogs; S: susceptible; SNP: single nucleotide polymorphism; SSR: simple sequence repeat; TF: transcription factor.

Importantly, WGS provides the foundation for genome editing technologies, such as CRISPR/Cas9, by enabling precise identification of target genes and regulatory sequences. In *C. sativa*, the first successful genome editing using CRISPR/Cas9 [166] is detailed in 2.4.2.1, demonstrating editing efficiency in protoplasts derived from somatic embryos. This DNA-free approach avoids transgene integration, offering a promising path for developing improved varieties without genetically modified organisms (GMOs) classification. The availability of reference genomes significantly enhances the accuracy and efficiency of such editing methodologies, allowing for targeted modifications that improve disease resistance, stress tolerance, and other agronomic traits.

In summary, WGS provides a foundational platform for integrating molecular genetics with ecological and evolutionary frameworks, accelerating both traditional breeding and cutting-edge genome editing approaches to develop resilient chestnut populations capable of withstanding current and emerging challenges.

2.3. Micropropagation Techniques

2.3.1. Axillary Budding Micropropagation

The initial objective of micropropagation techniques in European chestnut was to develop efficient systems for clonal propagation through axillary budding culture, using trees previously selected for their tolerance to *P. cinnamomi*. This method was conceived as a complementary alternative to the traditional propagation system based on ground-layering. European chestnut is a recalcitrant species, particularly with respect to rooting, a trait that persists even under in vitro conditions. To overcome these limitations, Spanish researchers initially optimized their methodology using juvenile plant material, which enabled the establishment of a reliable micropropagation system [167]. This protocol was later adapted for use with mature material, which in some cases required rejuvenation techniques (i.e., etiolation or repeated grafting) to elicit a suitable in vitro response [168–170]. Later on, numerous studies and reviews have been published in specialized journals on the in vitro propagation of European chestnut and its hybrids via axillary bud micropropagation [4,171–175]. Nowadays, micropropagation by axillary budding is an essential and strategic tool for the rapid, safe, and efficient cloning of superior chestnut genotypes with enhanced *P. cinnamomi* tolerance selected from European breeding programs. In Spain, a robust protocol that allows the efficient cloning of any European chestnut genotype was validated and transferred to the private sector through appropriate agreements, facilitating its application in commercial plant production [176]. For example, TRAGSA Company produced 40,000 plants in the most recent campaign, the first to be

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completed in the new laboratory, with the goal of testing the market's ability to absorb increased volumes. For the upcoming campaign, which is already underway, production is expected to rise to 50,000–60,000 plants. In 2025, the company plans to scale up production to 100,000 hybrid chestnut plants, intended for use as rootstocks. Meanwhile, CULTIGAR Company has produced an average of 58,000 plants per year over the past few seasons. In Portugal, micropropagation has played a critical role in producing homogeneous experimental material, fundamental for physiological, molecular, and pathogenicity assays conducted with high technical rigor [57,93,95] and in the efficient multiplication of rootstocks tolerant to *P. cinnamomi* [49](return to 2.1.1. for details on registered new chestnut varieties). Adapted for the transition from the laboratory to the field, a pilot facility was established in the North Alentejo region of Portugal, Marvão, equipped to produce thousands of clonal plants, ensuring uniformity, traceability, and phytosanitary quality. The location was chosen due to its chestnut-growing tradition and optimal edaphoclimatic conditions, serving as a demonstration and dissemination platform. Tolerance to *P. cinnamomi* observed under controlled conditions requires validation in the field. Experimental field trials were implemented to test the performance of new genotypes, evaluating parameters such as vegetative vigor, survival rate, productivity, and health over time. These results are crucial for assessing the commercial viability of new materials and informing technical recommendations to producers. In addition to disease tolerance, ensuring graft compatibility with traditional, commercially valued chestnut varieties is essential. Graft compatibility studies are being conducted to evaluate rootstock–scion interactions, considering factors such as affinity, development, precocity, and fruit quality. This approach protects regional genetic heritage while introducing resilience into production systems.

Factors such as plant vigor, rooting uniformity, and acclimatization performance were significantly improved, marking a major step toward the large-scale, energy-efficient production of elite European chestnut genotypes. A recent advancement is the use of LED-based systems, which have emerged as powerful tools to further enhance chestnut micropropagation. In this line, Marino et al. [177] demonstrated that specific LED spectra substantially improved both the multiplication and rooting phases, doubling the proliferation index and achieving 100% rooting success compared to conventional fluorescent lighting.

2.3.2. Somatic Embryogenesis

Somatic embryogenesis (SE) has also been established in European chestnut and its hybrids. However, these procedures cannot yet be applied at a commercial level. Despite this, they have been extremely useful for developing cryopreservation, genetic transformation, and gene editing methods in this species. The first well-documented report of true somatic embryo induction and subsequent plant regeneration from immature zygotic embryos of *Castanea sativa* × *C. crenata* hybrids was published by Vieitez et al. [178]. Later studies confirmed somatic embryogenesis in both hybrid (*C. sativa* × *C. crenata*) and pure *C. sativa* genotypes [179–183]. Somatic embryo induction typically involves a two-step culture protocol: an initial induction phase using auxins in high concentration, followed by an expression medium with either low concentrations of PGRs or none at all. Among auxins, 2,4-dichlorophenoxyacetic acid (2,4-D) is most commonly used and considered essential for initiating embryogenic cultures. A cytokinin, usually benzyladenine (BA), kinetin, or zeatin, is often added at lower concentrations to support embryo development. Particulary, Sezgin and Dumanoğlu (2014)[181] successfully used indole-3-butyric acid (IBA) in combination with thidiazuron (TDZ), as an alternative induction treatment. The developmental stage of the zygotic embryo, closely tied to the collection time (weeks post-anthesis), strongly affects induction efficiency. The optimal window for harvesting embryos in northwest Spain ranges from 6 to 12 weeks post-anthesis (late July to early September), when immature zygotic embryos are most competent for somatic embryogenesis. Both whole zygotic embryos and cotyledonary segments have been used as explants. However, comparative studies indicate that embryonic axes exhibit twice the induction efficiency of cotyledonary pieces [179]. Moreover, the induction percentages were influenced by both the

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genotype of the mother tree and collection date, with values ranging from 2% for the hybrid material [178,183] to 11.1% for *C. sativa* material [179,182].

To date, SE from explants other than zygotic embryos in chestnut species has only been successfully achieved in European chestnut by Corredoira et al. [179,184]. Somatic embryos were induced from the most apical leaves excised from proliferating shoot cultures, using a three-step culture protocol. In the first step, explants were cultured on medium supplemented with 4 mg/L naphthaleneacetic acid (NAA) and 0.5 mg/L BA. This was followed by subculturing onto a medium containing reduced concentrations of both PGRs at 0.1 mg/L, and finally transferring to a PGR-free expression medium. Notably, 2,4-D and IBA regulators, commonly effective in the SE induction from zygotic embryos, were ineffective in inducing embryogenesis from non-zygotic-derived explants. Induction rates were considerably lower than those obtained from zygotic embryos, with frequencies not exceeding 1% [179]. However, subsequent studies significantly increased induction frequencies through the incorporation of 2 mg/L larch wood extract, a compound rich in arabinogalactan proteins (AGPs), resulting in SE induction rates of up to 5.3%—the highest reported to date for non-zygotic explants in European chestnut [185].

Another critical challenge in the application of SE in European chestnut, as in many other woody species, is achieving efficient plant regeneration, defined as the simultaneous development of both shoot and root systems from somatic embryos. The successful maturation and conversion of somatic embryos into viable plantlets are influenced by several factors, including the type of osmotic agents used, pre-germination treatments, and the application of PGRs [35]. In the European chestnut, the source and concentration of carbon are particularly important. Among the tested sugars, 3% maltose yielded the best results, with an overall plant recovery rate of 39%, including 6% of embryos achieving full plantlet conversion and 33% showing shoot development only [184]. In addition, increasing the agar concentration to 1.1% in the maturation medium significantly improved embryo maturation and plantlet conversion rates (10–25%) in embryogenic lines derived from zygotic embryos **[180]**. In contrast, ABA, which is commonly used to promote maturation in other embryogenic systems, proved largely ineffective in chestnut, showing no significant improvement in plantled development or conversion [183]. Direct transfer of somatic embryos from maturation to germination media often results in poor germination and abnormal plantlet development. Therefore, pre-germination treatments such as cold storage, desiccation, and the application of gibberellic acid (GA_3) are typically required to improve conversion outcomes [35]. In European chestnut, application of cold storage of 2 months somatic embryos provided conversion rates of up to 38.9% in C. sativa lines [184] and 29–32% in hybrid material [183,186]. Desiccation, which mimics the natural drying phase in seed development, has also proven beneficial. Fast desiccation by exposing embryos for 2 h in a laminar flow hood reduced moisture content to approximately 57%, resulting in significantly enhanced plant quality [187]. Similarly, **Sezgin & Dumanoğlu [182]** achieved 40% plant regeneration using cold storage followed by slow desiccation, applying a desiccator with saturated salt solution. In the germination step, somatic embryos are generally cultured on germination media, often supplemented with low concentrations of cytokinins alone or in combination with auxins. For instance, the inclusion of 0.1 mg/l BA together with 0.1 m/l NAA or 0.1 mg/l IBA yielded the highest conversion rates **[181,187]**. Further improvements in plantlet development were achieved by adding 200–438 mg/L glutamine or 150 μM Fe-Na-EDTA to the germination medium [183,187]. Despite these advancements, plant regeneration remains a bottleneck in chestnut SE protocols, particularly due to the asynchronous or incomplete development of shoots and roots, underscoring the need for further optimization of both culture conditions and genotype-specific responses.

2.4. Genetic Engineering Strategies

One of the greatest advances in plant breeding has been achieving modified plants through genetic transformation by inserting specific genes into the plant genome. A fundamental premise for success in the production of transgenic plants is the availability of an in vitro regeneration system adequate to support the production of plants from cells, organs, or tissues susceptible to infection by

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Agrobacterium tumefaciens. Traditional genetic transformation and modern genome editing technologies have been employed in European chestnut for research and breeding purposes using somatic embryogenesis as regeneration system. Remarkably, it stands out as one of the first forest tree species in which gene editing has been successfully implemented, representing a significant advancement in the genetic improvement of woody plants.

2.4.1. Traditional Genetic Transformation

The first attempt at genetic transformation in European chestnut was conducted by Seabra and Pais [188], using *Agrobacterium*-mediated transformation of hypocotyl segments excised from in vitro-germinated seedlings. The bacterial strain LBA 4404 was employed, carrying the binary vector p35SGUSINT, which includes the *nptII* gene (conferring kanamycin (Kan) resistance) and the *uidA* (GUS) as a reporter gene, allowing detection of transgene expression through β-glucuronidase activity assays. Although transgenic shoots were regenerated, molecular analyses revealed that many were chimeric, indicating incomplete or partial transformation events. Successful stable genetic transformation was later achieved using somatic embryos as target explants, employing the same marker genes *nptII* and *uidA* [189,190]. In the initial transformation experiments, the influence of *Agrobacterium* strain/plasmid combinations and co-cultivation duration (3 vs. 4 days) was assessed [189]. After 12 weeks on selective medium, 112 out of 624 explants were kanamycin-resistant. Transformation efficiency, defined as the percentage of explants producing GUS-positive embryogenic cultures, was significantly affected by both the bacterial strain/plasmid and co-culture period. The highest transformation efficiency (25%) was obtained using strain EHA105 harboring plasmid pUbiGUSINT after 4 days of co-cultivation. Subsequently, the same authors investigated the effects of acetosyringone addition, bacterial density, genotype, and the developmental stage of somatic embryos on transformation efficiency [190,191]. Contrary to expectations, acetosyringone at both 100 μM and 200 μM negatively impacted transformation efficiency. All tested bacterial densities yielded kanamycin-resistant embryos, with the highest transformation efficiency (20.1%) observed when *Agrobacterium* was in the exponential growth phase ($OD_{600} \approx 0.6$); however, the differences between densities were not statistically significant. Both the developmental stage of the somatic embryos and the genotype had significant impacts on transformation success. Globular and heart-shaped embryos, as well as embryo clumps, exhibited higher transformation frequencies (up to 30%) compared to cotyledonary-stage embryos, likely due to their higher proliferation potential and a greater proportion of actively embryogenic cells [192]. Genotype-dependent variability was also evident: among seven lines tested, two showed relatively high transformation efficiencies (21.7% and 33.8%), while the others ranged between 1.7% and 10%. Molecular analyses confirmed transgene integration, with all GUS-positive lines testing PCR-positive for *uidA* and *nptII*. Southern blot analysis further verified uidA presence in transgenic lines, but not in untransformed controls.

Once an efficient transformation method had been established, the next step was to introduce genes with the potential to enhance disease resistance. To this end, two PR proteins—a thaumatin-like protein (CsTL1)[193] and a chitinase (CsCH3)[125], which had been purified from mature cotyledons of European chestnut and shown to exhibit significant antifungal activity in vitro, were selected for gene overexpression (section 2.2.2.1). Somatic embryos were transformed using *A. tumefaciens* strain EHA105 carrying a binary plasmid containing the *CsTL1* gene, the *nptII* selectable marker, and the *egfp* reporter gene [194]. The *CsTL1* gene was successfully overexpressed in three somatic embryogenic lines, with transformation efficiencies ranging from 7.1% to 32.5%, resulting in the generation of 126 independent transgenic lines. Building on this work, an efficient *Agrobacterium*-mediated protocol was later developed to introduce the endogenous *CsCH3* gene into somatic embryos, further advancing efforts to enhance disease resistance in European chestnut [126]. A total of 88 independent chitinase transgenic lines were obtained. In both studies, stable integration of *CsTL1* and *CsCH3* genes into the genome was confirmed by PCR and Southern blot analysis. qPCR further showed that *CsTL1* expression was up to 13.5-fold higher in one transgenic line compared to its untransformed counterpart. GFP fluorescence, indicative of transgene activity, was observed not

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only in somatic embryos but also in shoots, leaves, and roots of transgenic plants. Importantly, no phenotypic differences were detected between transgenic and control plants, suggesting that GFP expression and transgene integration did not produce any pleiotropic effects. These molecular and phenotypic results support the potential of *CsTL1* and *CsCH3* as candidate genes for engineering resistance to ink disease in European chestnut. Nevertheless, further functional validation under greenhouse and field conditions is essential to confirm the effectiveness of these transgenes against *Phytophthora* sp and/or *C. parasitica* infection in planta.

Using the same transformation system, twelve independent transgenic European chestnut lines were obtained carrying the already mentioned *Cast_Gnk2-like* gene (section 2.2.1), which encodes a Gnk2-like antifungal protein. Transgene copy number and relative expression were analyzed by qPCR in lines with high proliferation capacity [101]. Tolerance assays against *P. cinnamomi* revealed that one transgenic line exhibited significantly improved tolerance compared to the non-transformed control, as evidenced by reduced root necrosis and consequently fewer disease symptoms [195].

Adapted systems of genetic transformation of somatic embryos and plant regeneration have been applied to *Cast_Gnk2-like* functional validation in *C. dentata* [101,104], *Quercus ilex* [102] and *Q. suber* [103]. Tolerance assays to *P. cinnamomi* are ongoing for *C. dentata*, while oak plantlets overexpressing *Cast_Gnk2-like* potentially exhibit improved tolerance to *P. cinnamomi*, compared to non-transformed plantlets. As oaks belong to the same family as chestnuts and both are susceptible to *P. cinnamomi*, these results are a promise for Fagaceae improvement. To further support *Cast_Gnk2-like* as a gene conferring tolerance to *P. cinnamomi*, the recombinant protein expressed in *Escherichia coli* showed activity against *P. cinnamomi* in laboratory pathogenicity tests, validating its potential as a protective agent and opening perspectives for the use of bioactive compounds in integrated disease management [196]. These approaches demonstrate the utility of genetic engineering for functional validation, a deeper understanding of resistance mechanisms, and the development of innovative plant protection tools.

Plant regeneration, defined as the development of both shoot and root from transgenic somatic embryos, was consistently low, irrespective of genotype. These low conversion rates significantly limit the number of plantlets available for downstream analyses. However, some somatic embryos undergo partial germination, producing shoots without roots. These shoots can be leveraged through axillary shoot proliferation and subsequently induced to root, allowing for the propagation of an unlimited number of transgenic plantlets [197]. This method provides sufficient plant material for essential molecular validation and for performing disease resistance assays. Although improving the direct conversion efficiency of somatic embryos into complete plantlets remains a key objective, axillary shoot culture currently stands as the only reliable and scalable strategy for multiplying transgenic chestnut lines for research and functional evaluation.

2.4.2. New Plant Breeding Techniques

New Plant Breeding Techniques (NPBTs) are modern biotechnological tools that enable precise modification of the plant's DNA without introducing genes from other species. Unlike conventional breeding, which requires multiple cycles of crossing and selection, NPBTs can directly modify elite cultivars, reducing breeding timelines and minimizing the risk of losing valuable traits during hybridization [198]. This is particularly relevant for woody species, characterized by long generation times and high heterozygosity level.

Among NPBTs, the CRISPR/Cas9 (*Clustered Regularly Interspaced Short Palindromic Repeats-Cas9*) system is the most widely applied genome editing tool in plant biotechnology, thanks to its versatility and the ability to target multiple genes simultaneously [199]. The system takes advantage of a guide RNA (gRNA) to direct the Cas9 nuclease to a specific DNA sequence, where it generates a double-strand break (DSB). The plant cell repairs this break mainly through non-homologous end joining (NHEJ), which often introduces small insertions or deletions that disrupt the gene function, or less frequently, through homology-directed repair (HDR), which can introduce precise sequence changes if a donor DNA template is provided [200].

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In woody species, CRISPR/Cas9 applications are still limited [201]. As in the Traditional transformation, the main challenge is plant regeneration: edited cells must be able to develop a whole plant, a process that is often inefficient and highly genotype-dependent [174,177]. In *C. sativa*, SE remains the most effective regeneration system, offering higher transformation efficiency and reducing the occurrence of chimeric plants [35,179].

An advancement over standard CRISPR/Cas9 delivery is DNA-free genome editing using ribonucleoprotein (RNP) complexes, in which purified Cas9 protein is pre-assembled with its gRNA and introduced directly into protoplasts, plant cells deprived of the cell wall [202]. In conventional vector-based systems, Cas9 can remain active for a long time, increasing the risks of off-target mutations. In contrast, RNPs act only for a short time before being degraded by the cell, limiting nuclease exposure and avoiding the stable integration of foreign DNA [198]. This non-integrative approach enables the generation of edited plants completely free of recombinant DNA, opening new opportunities for breeding and potentially improving consumer acceptance compared with traditional GMOs. RNPs can be delivered by particle bombardment, protoplast electroporation, or polyethylene glycol (PEG)-mediated uptake but their application in woody crops remains limited. The main challenge is that efficient delivery must be combined with efficient regeneration from edited cells, which continues to represent a major bottleneck for *C. sativa* and many other woody species [203].

2.4.2.1. CRISPR/Cas9 Genome Editing in Castanea Sativa

The first successful application of CRISPR/Cas9 in European chestnut was reported by Pavese et al. [204] as a proof-of-concept for targeted mutagenesis in this recalcitrant woody species. The phytoene desaturase (*pds*) gene, involved in carotenoid biosynthesis, was selected as it serves as a visible marker, producing an easily recognizable albino phenotype caused by carotenoid loss and chlorophyll degradation. Two single-guide RNAs (sgRNAs) were designed to target conserved exonic regions of *pds* and assembled into a binary vector containing *Cas9* and the *nptII* selectable marker for Kan resistance. Transformation was performed on somatic embryos at globular and torpedo stages, produced according to established protocols [173,179], using *A. tumefaciens* strain EHA105. Following co-cultivation, embryos were cultured on Kan-containing medium to select the Kan-resistant ones. Four kan-resistant embryogenic lines were recovered, and sequencing of the target region confirmed the editing event and the presence of small insertions and deletions. Regenerated plantlets from edited lines displayed the expected albino phenotype, providing direct phenotypic validation of *pds* knockout. This study demonstrated that CRISPR/Cas9 can be applied in *C. sativa* using SE as starting material. More recently, this system has been extended to genes of agronomic relevance. In particular, susceptibility genes are being targeted to improve tolerance to *P. cinnamomi* [107], with the goal of developing chestnut genotypes with enhanced disease resistance, improved nut quality, and better stress tolerance.

2.4.2.2. DNA-Free Genome Editing Using Ribonucleoproteins (RNPs)

Pavese et al. (2022) [166] realized the first protocol for DNA-free genome editing in European chestnut, using pre-assembled Cas9–sgRNA ribonucleoprotein (RNP) complexes delivered into protoplasts derived from somatic embryos. High yields of viable protoplasts (~4.5×10^6/mL with 91% viability) were obtained using enzymatic digestion with cellulase (1%) and macerozyme (0.5%) for 4 h of incubation. Transfection efficiency was confirmed using a GFP marker, with ~51% of protoplasts expressing GFP 72 h post-transfection. Finally, RNPs targeting *pds* were directly delivered, and sequencing confirmed successful editing of the target gene. This work demonstrated that RNP-mediated editing is feasible in *C. sativa* and provides an important step toward precise, DNA-free editing in chestnut. The main limitation is still the regeneration of complete plants. To move from edited protoplasts to improved chestnut genotypes, RNP delivery must be combined with efficient somatic embryogenesis, which is crucial for developing traits such as pathogen resistance and stress tolerance.

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2.5. Germplasm Conservation Through Cryopreservation

Cryopreservation is a technique that allows for the long-term conservation of biological tissues—such as embryos, seeds, shoot tips, or pollen at ultra-low temperatures, typically in liquid nitrogen (-196 °C, LN) [205]. At this temperature, all cellular metabolic activity comes to a complete halt, enabling the indefinite preservation of biological material. This approach is a crucial tool for conserving genetic diversity, particularly in species with recalcitrant seeds that are sensitive to extended storage periods, as is the case with chestnut species. In European chestnut, cryopreservation serves as a valuable complement to traditional field collections for the long-term conservation of germplasm, and it has been successfully applied to a wide range of materials, including zygotic embryos, somatic embryos, and axillary shoot tips excised from shoots maintained in vitro [171].

Remarkably, Corredoira et al. [206] developed a highly efficient protocol for the cryopreservation of zygotic embryos. This study involved isolating embryonic axes and subjecting them to various desiccation periods (ranging from 0 to 7 h) prior to immersion in LN. Moisture content was found to be a critical factor influencing post-cryopreservation survival. At the time of isolation, the embryonic axes had a moisture content of 65.7% (based on fresh weight), which progressively decreased to 18% after 7 h of desiccation in the laminar flow hood. The highest survival rate (100%) and the best plant regeneration rate (63%) were achieved when the moisture content was approximately 20%, reached after 5 h of desiccation. Later, Gaidamashvili et al. [207] achieved a comparable whole-plant regeneration rate of 64% using an encapsulation with activated charcoal-vitrification protocol, which is a significantly more complex and labor-intensive procedure.

Significant progress has been made in recent years in the cryopreservation of somatic embryos in woody species, including European chestnut [205]. Vitrification-based cryopreservation has been successfully applied to chestnut somatic embryos using a protocol that combines a 3-day preculture on 0.3 M sucrose medium, treatment with Plant Vitrification Solution 2 (PVS2)[208] for 60 min at 0 °C, and rapid immersion in LN. Embryo clumps at the globular or heart stage achieved a 68% recovery rate, which remained stable even after 10 years of cryostorage (Corredoira, unpublished data), confirming the long-term reliability of the method. Moreover, this approach was successfully applied to the cryopreservation of transgenic embryogenic lines carrying a thaumatin-like protein gene [194] and a chitinase gene [126], underscoring its value for preserving genetically modified material during tolerance trials. Desiccation-based cryopreservation has been investigated as an alternative to vitrification for chestnut somatic embryos; however, it has produced poorer results. While vitrification methods obtained embryo recovery rates ranging from 68% to 92%, desiccation protocols resulted in only about 33% recovery. This highlights the greater efficiency of vitrification-based techniques for cryopreserving chestnut embryogenic tissues [194,206].

Shoot tips isolated from in vitro shoot cultures were also cryopreserved following the optimization of several parameters, including explant type, PVS2 exposure duration, sucrose concentration in the preculture medium, loading pre-treatment, and the sequence of recovery media used after thawing [209]. The most effective shoot regrowth protocol utilized 0.5–1.0 mm shoot tips that had been cold-hardened for two weeks. After a two-day preculture on medium supplemented with 0.2 M sucrose, the shoot tips underwent a two-step vitrification procedure: first, 20 min at room temperature in a loading solution (2 M glycerol + 0.4 M sucrose), followed by 120 min at 0 °C in a modified PVS2 solution, before rapid immersion in LN. This protocol resulted in 38–54% shoot recovery across five chestnut clones (of both juvenile and mature origin), with successful plant regeneration achieved in all cases. Later on, this protocol enabled, for the first time, the establishment of a European chestnut germplasm bank, in collaboration with the public company TRAGSA, aimed at conserving *P. cinnamomi*-tolerant genotypes [210]. Of the 46 chestnut genotypes tested, 43 survived cryopreservation, although only 63% retained their capacity to regenerate new shoots.

3. Conclusions and Future Perspectives

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Climate change affects European chestnut populations by altering their phenology, increasing drought and heat susceptibility, which impacts growth, fruit quality, and survival. Warmer and wetter conditions also increase the spread of diseases and pests, increasing tree vulnerability. In this context, technologies such as traditional genetic transformation, omics-based approaches, gene editing (e.g., CRISPR/Cas9), in vitro culture and cryopreservation provide a powerful and complementary toolkit for modern breeding. These biotechnological tools enable the precise introduction of disease resistance, characterization of genetic diversity, conservation and the targeted enhancement of key agronomic traits, ultimately contributing to the development of more resilient, productive, and sustainable European chestnut cultivars.

Micropropagation through axillary bud proliferation is a well-established technique, widely applied at the commercial level by numerous companies. However, the development of embryogenic systems from non-zygotic material, particularly that derived from mature genotypes, will require considerable efforts in the coming years to achieve optimization. Over the years, development of somatic embryogenic protocols has been refined through empirical approaches, involving the testing of various culture media, growth conditions, donor plant ages, and explant types. However, to further advance in vitro plant cell reprogramming and regeneration, new strategies are needed to identify novel chemical promoters that can enhance these processes [211]. Although European chestnut is one of the few species for which efficient cryopreservation protocols have been developed for various tissues, its practical application remains very limited. Given the current context of climate change and the pressing need to preserve biodiversity, efforts to establish cryobanks should be strongly promoted, especially by public institutions.

Traditional *Agrobacterium*-mediated transformation has been an effective tool for introducing genes into embryogenic tissues, enabling traits such as disease resistance and stress tolerance. More recently, gene editing technologies like CRISPR/Cas9 have enabled precise modifications of endogenous genes without the need to insert foreign DNA, offering a more targeted and socially acceptable alternative. Although still in the early stages of application in chestnut, gene editing holds great promise for accelerating the development of improved genotypes, particularly in addressing diseases. Looking ahead, the availability of both traditional transformation and genome editing procedures provides a comprehensive platform for the genetic breeding of European chestnut, paving the way for more resilient and sustainable cultivation systems. On the other hand, both tools are convenient for functional analysis and validation of genes of interest.

The advances in omics technologies particularly genomics, transcriptomics, and metabolomics have significantly deepened our understanding of European chestnut, enabling the identification of key genes associated with important agronomic traits such as disease resistance, fruit quality, and adaptation to abiotic stress. An integrative approach combining transcriptomics, proteomics, and metabolomics would underscore the multifaceted nature of the defense response and highlight specific molecular deficits in susceptible chestnut species that may be targeted for improvement. Besides the contribution to ongoing efforts in MAS for chestnut controlled and expedited breeding, the expanding repository of genetic and functional information creates new opportunities for applying gene editing tools with greater precision and efficiency, targeting multiple well-characterized genes. Consequently, genome editing approaches that combine resistance allele stacking in breeding strategies with the modification of susceptibility genes will accelerate the development of elite chestnut varieties adapted to the challenges of climate change, with enhanced biotic stress resilience, productivity, and sustainability. This synergy between biotechnology and omics science positions the European chestnut at the forefront of a new era of applied genomic innovation.

New chestnut varieties that result from the improvement programs to pests and diseases need to be tested on susceptibility to climatic extremes (i.e., heat waves, prolonged droughts, and late frosts) to evaluate genotype resilience in the context of climate change projections.

Taken together, the studies here reported illustrate how molecular biotechnology approaches from population-level SSR diversity analysis to transcriptome-based marker development and gene

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expression profiling are converging to advance chestnut research. By linking insights into genetic diversity, molecular markers, and defence mechanisms, they pave the way for more informed strategies to conserve unique germplasm and breed cultivars resilient to diseases and pests.

The integration of science, technology, and commercial application is essential to address the challenges facing chestnut cultivation in the 21st century. The research programs on chestnut conducted in Portugal, Spain, Italy, and the US (from the development of more tolerant genotypes to their validation and large-scale production in real-world conditions) exemplify knowledge transfer with direct impact on agricultural sustainability, the valorization of mountain territories, and the preservation of an emblematic resource of rural landscape and culture. These programs highlight the critical role of applied research as a driver of innovation, regional development, and climate adaptation.

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