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# Functional identification of CmAP1, the homologous gene of APETALA1, in *Castanea mollissima*

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Abstract

The MADS-box gene *APETALA 1* (*AP1*) plays essential roles in the processes of floral initiation and floral organ development. Here, the coding sequence (CDS) and promoter of *CmAP1*, which is the homologous gene of *AP1*, were cloned from Chinese chestnut (*Castanea mollissima*) “Yanshan Hongli”. The CDS of *CmAP1* was 741 bp length and encode a 346 amino-acid protein. Subcellular localization analysis revealed that *CmAP1* is localized in the nucleus. *CmAP1* was found to be mainly expressed in inflorescence and the expression level of *CmAP1* gradually increased as inflorescence developed. *CmAP1* promoter drove the expression of *GUS* in leaf margin water holes, trichomes, flower stalk ends, carpels, and immature pods. The auxin response element (TGA element), jasmonic acid response element (TGACG-motif), and WRKY binding site (W-box element) were all located in the -645 to -285 region of *CmAP1* promoter, which is essential for the expression of *CmAP1* in the carpels and immature pods of *Arabidopsis thaliana*. Over-expression of *CmAP1* in *A. thaliana* increased the expressions of *FLOWERING LOCUS T* (*AtFT*), *AtAP1*, and *LEAFY* (*AtLFY*), and promoted flowering. The floral development was not affected by the over-expressed *CmAP1*. These

results suggest that *CmAP1* participates in flowering. The role of *CmAP1* in the development of carpel and fruit need further research.

Keywords: *Castanea mollissima*; APETALA1; promoter; transgenic; flowering

# Pooled whole genome sequencing method (Pool-Seq) to identify genomic regions associated to *Dryocosmus Kuriphilus* Yasumatsu resistance in European *Castanea sativa* Mill

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## Abstract

The insect *Drycosmus kuriphilus* (Asian chestnut gall wasp) was accidentally introduced from China to Italy in 2002, and has become one the most damaging pests of *Castanea sativa* (sweet chestnut). It infests chestnut buds, inducing formation of galls, with strong impact on plant growth and nut production. The main strategy to control *D. kuriphilus* has been the introduction and massive release of the parasitoid *Torymus sinensis* as biocontrol agent. On the opposite, little effort has been made to improve the resistance of the host plant.

A comparative field trial at IRET-CNR, including provenances from Spain, Italy, and Greece, was screened for severity of infestation by *D. kuriphilus*. The attacks of the gall wasp were measured in the plantation

as a percentage of bud infestation on winter branches and on spring shoots. A great diversity among plants and a significant heterogeneity among the geographical provenances were found. Two independent studies identified the Greek provenance “Hortiatis” that expressed the lowest susceptibility and the highest proportion of immune plants. The phenotypic data suggest that this provenance could bring specific genetic factors of resistance to *D. kuriphilus*. It represents a good plant material for a Pool-Seq experiment to perform a genome-wide association study by comparing susceptible and resistant plants.

The experimental design included susceptible and resistant plants from the provenance “Hortiatis” to reduce the risk of spurious association due to population structure. DNA pools of the two groups (25 plants each) were sequenced with 50X coverage depth. Sequence reads were aligned to the reference genome of *C. mollissima* and the two pools were compared to identify SNPs associated to resistance thanks to PoPoolation2 software, which allows to identify significant differences in SNP allele frequencies between two or more populations. Genomic regions containing the significant SNP differences will be associated to low plant susceptibility and will be screened to identify candidate genes and clarify the plant resistance mechanisms. After validation in unrelated independent plant material, the resistance-associated SNPs could be developed into selection markers for breeding programmes.

Keywords: Genome-Wide Association Study; Asian Chestnut Gall Wasp; Pool-seq

## *Castanea crenata* Ginkbilobin2-like as a resistance gene to *Phytophthora cinnamomi* infection

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## Abstract

Many species of the *Fagaceae* family are susceptible to diseases caused by the oomycete *Phytophthora cinnamomi*. Comparative transcriptomics with resistant (Japanese) and susceptible (European) chestnut species, identified several candidate resistance genes to this pathogen. Among these genes is a Ginkbilobin2-like, which encodes for an anti-fungal protein and is putatively related to constitutive and induced defenses due to its high expression levels in Japanese chestnuts before and after *P. cinnamomi* inoculation. Genetic transformation and protein analysis approaches are ongoing to validate *Cast_GNK2-like* as a resistance gene to *P. cinnamomi*. Susceptible European and American chestnuts, Holm and Cork oaks have been genetically transformed to overexpress *Cast_GNK2-like*. Transformants were successfully obtained for all species. Characterization of the lines (copy number, gene expression) and inoculation assays are currently ongoing. Holm oak plants overexpressing *Cast_GNK2-like* demonstrated a delay in pathogen progression after in vitro inoculation assays. The encoded protein has been expressed in heterologous systems to be isolated and used in confrontation assays with *P. cinnamomi*. Preliminary results point to a pathogen lag in the presence of the protein. The multidisciplinary approach in progress to functionally characterize *Cast_GNK2-like* is revealing the strong potential of the gene to be used as a molecular marker to select genotypes in breeding programs and the potential of the protein as an anti-oomycete compound. The outcomes of this work will further our knowledge of the mechanisms underlying the response to *P. cinnamomi* and contribute to developing pathogen control strategies.

Keywords: Chestnut; Oak; Ink disease; Oak decline; Cysteine-rich repeat secretory protein

## The first european chestnut (*C.sativa*) genome

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## Abstract

Sweet chestnut (*Castanea sativa*) is a member of the Fagaceae family producing edible nuts that represent an economically important product for Europe with an annual production of 240.000 tons between 2016 and 2020 (FAOSTAT 2022). To further valorize and improve the European Chestnut germplasm, a deep study of the *C. sativa* genome and genomic diversity is essential. To date there is no genomic data available for *C. sativa* while there are two draft genomes of *C. mollissima.*

Here we present the first chromosome scale assembly of sweet chestnut cultivar 'Marrone di Chiusa Pesio'. The assembly was produced using a hybrid sequencing strategy that combined a high coverage (100X) of long (Oxford Nanopore Technology) and short (Illumina) reads, paired with chromosome conformation capture (Dovetail Omni-C). The total size of the assembled genome is 750.8 Mbp for a total of 238 molecules. The N50 is 25.0 Mbp in 12 scaffolds and the N90 is 2.9 Mbp in 42 scaffolds. The scaffolds have been anchored and oriented in 12 pseudo-chromosomes using a dense genetic map derived from a cross between 'Madonna' and 'Bouche de Betizac' (Torello Marinoni et al, 2020).

The release of the first reference genome will enable the study of chestnut genetics at a genome-wide scale providing genes useful for targeted breeding and the basis to untangle complex phenotypic pathways. Moreover this genome can be used in resequencing activities to identify allele variants to produce a set of evenly spread and high-quality SNPs to facilitate genetic studies in the Castanea genus.

Keywords: Chestnut genome, *Castanea sativa*, genome

# A comparative transcriptomic analysis of low boron-efficiency and high boron-efficiency *Castanea mollissima* varieties

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## Abstract

Chinese chestnut (Castanea. mollissima Blume; Fagaceae) is one of the five famous fruits in ancient China, along with jujube, peach, apricot and plum. It is also one of the earliest fruit trees cultivated in China, and has the reputation of “king of dried fruits”, because it has a delicious taste and rich nutrition. Empty shell is one of the important factors affecting chestnut yield. There are many reasons for empty shell formation. At present, it is believed that it is mainly related to tree body nutrition status, poor pollination and fertilization, embryo development obstruction and other factors. Many studies believe that boron deficiency is the main cause of chestnut empty shell. Boron fertilizer application can effectively increase chestnut yield. Therefore, low-boron-tolerant chestnut varieties are one of the important directions for the development of chestnut industry in China.We have developed two varieties, one resistant to low boron(Named H7) and the other sensitive to boron(Named YH). In this study, we conducted transcriptome analysis on these two varieties. A total of 58 samples from buds, leaves, flowers, and fruits were taken in three periods and processed for transcriptome sequencing, generating 354.56Gb Clean Data. Differentially expressed genes (DEGs) were identified using the criteria of Fold Change≥2 and FDR < 0.01. Then GO/KEGG enrichment, gene set enrichment analysis(GSEA), differential alternative splicing (AS) and protein interactions of DEGs were analyzed. We found 1,292 differentially expressed genes, of which 580 were up-regulated and 712 were down-regulated. Differentially expressed genes were mainly enriched in the following pathways :plant-pathogen interaction, starch and sucrose metabolism, protein processing in endoplasmic reticulum and spliceosome. This study will contribute to the development of molecular markers for screening low boron tolerant chestnut varieties in the future.

Keywords: Chestnut, Boron efficiency,transcriptomic analysis

# Genes activated during the defensive response of resistnat chestnut genotypes againts *Dryocosmus kuriphilus*


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## Abstract

After the invasion of the Asian gall wasp *Dryocosmus kuriphilus,* several chestnut genotypes (*Castanea sp*.) were described as resistant to this species. Studies of the defensive mechanism involved in this process revealed that the immune response of the resistant genotypes rely mainly in a hypersensitive reaction (HR) to eggs or neonate larvae within the tree buds. HR is an induced resistance response that can be detected in the area immediately adjacent to the egg mass within the tree bud and show up as a necrotic spot preventing gall formation. HR is triggered by specific molecules, called elicitors, responsible for the activation of the defensive response and involves several steps: recognition of insect, development of oxidative burst, activation of defense genes, and cell death. In the present work we identify the defensive genes involved in this process through gene expression analysis. We performed RNAseq which enables the sequencing of mRNA from different phenotypes and experimental conditions for a highly sensitive and accurate evaluation. This technology allows us to identify differences in the defensive response involving immune-related genes between sensitive and resistant plants. The aim of this research is to improve the understanding of the mechanisms underlying the immune response in plants for further practical applications to control this parasite.

Keywords: gall wasp, hypersentitive reaction, elicitors, defensive response

# A specific molecular diagnostic test for *Gnomoniopsis castanea* (syn. *smithogilvyi)* and *Cryphonectria parasitica*

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## Abstract

Two fungi cause chestnut tree diseases in Switzerland: *Cryphonectria parasitica*, the endemic chestnut canker agent, and *Gnomoniopsis castanea (*syn. *G. smithogilvyi*) an endophytic fungus, recently identified in Europe and Switzerland as the main agent of chestnut fruit brown rot, also causing chestnut canker. The latter seems to cause a high mortality in young chestnut nurseries and orchards. In order to evaluate the presence of these fungi in the plant material used for the multiplication of 6 varieties of chestnut trees in Ticino, specific molecular diagnostic tests were developed for both species. All sequences available in GenBank for the internal transcript spacer (ITS) of the ribosomal DNA, the elongation factor 1-alpha (EF1a) gene and the beta-tubulin gene (TUBB), were collected for these two fungi. Significant differences between *Gnomoniopsis castanea*, *Gnomoniopsis spp.* and *C. parasitica* were sought. After analysing 164 ITS, 90 EF1a and 45 TUBB sequences, only the TUBB gene sequences showed any significant differences between both species. Specific PCR primers for each species were then designed from the TUBB sequences alignment. *In silico* analyses with BLAST (GenBank) confirmed the strict specificity of these primers. The two primer pairs were then tested with DNA extracted from previously characterised isolates of *G. castanea* and *C. parasitica* from Ticino, Wallis and Geneva, from roots and stems of germinated chestnuts or leaves of chestnut trees. These tests showed great robustness and represent an interesting tool to describe the

phytosanitary status of propagation material, especially for *G. castanea*, which is an endophytic fungus.

Keywords: *Gnomoniopsis castanea, G. smithogilvyi, Cryphonectria parasitica*

## Genomic approaches to dissect drought tolerance in *Castanea sativa* Mill

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Abstract

Climate change poses a significant threat to forest systems by intensifying the frequency and severity of drought events and heat waves, thereby accelerating the decline of European chestnut (*Castanea sativa* Mill.). This is especially concerning given the wide distribution of *C. sativa* in the Mediterranean basin, which encompasses regions characterized by strong gradients in water availability and genetic diversity correlated with drought tolerance. Unfortunately, the genetic study and identification of genes that regulate drought tolerance in chestnut has received little attention. Understanding the genetic basis underlying drought tolerance traits is crucial to develop effective management and conservation of *C. sativa*.

This study is based on knowledge on drought tolerance acquired from other forest species and model plants (such as *Populus* and *Quercus* spp.). The high degree of synteny between the two genera makes these results particularly relevant for chestnut. Additionally, the availability of the reference genome of *C. mollissima* and *C. crenata*, along with public sequences of *C. sativa*, is a valuable resource for comparative genomics studies. Our goal is to

create a comprehensive genome-wide atlas of gene families involved in pathways controlling this trait using computational analyses. We will also provide functional annotation based on Gene Ontology and a physical map, offering new insights into genomic regions responsible for drought tolerance in chestnut. Ultimately, the discovery and analysis of genes governing these traits can have important implications for chestnut breeding programs and forest planning. Additionally, this study provides a framework for further comparative genomic analysis and the evolution of drought-tolerant genes within the Fagaceae family.

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Keywords: abiotic stress, BLASTp, candidate genes, European chestnut, orthology

## Chromosome-level genome assembly of the red mutant of Chinese chestnut（*Castanea mollissima*）provides new insights into acumination of anthocyanin

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Abstract

The new leaves and spines of the red mutant of Chinese chestnut are red in spring, and the spines gradually turn red with maturation. In order to investigate the generation, evolution and inheritance of red Chinese chestnut mutants, we sequenced and assembled the genome of mutants, and combined the transcriptome and metabolome to analyze the source of differences and find the key sites of differences. Here, we

presented a high-quality chromosome-level reference genome of the red Chinese chestnut by combining PacBio HiFi reads and Hi-C sequencing technologies. The final assembly consists of two haplotypes, 706.0 Mb for hap1 and 702.8 Mb for hap2, and BUSCO analyses show complete gene percentage of 97.4% for hap1 and 97.2% for hap2. A total of 402.86 Mb of repetitive sequences for hap1 and 404.91 Mb for hap2, respectively. After genome annotation, 51,677 gene models for hap1 and 51,007gene models for hap2 were predicted in these two haplotypes. Transcriptome sequencing and anthocyanin metabolism were used to analyze the differential gene expression associated with different leaf colors and spiny bud growth stages and the relationships between gene expression and anthocyanin contents. KEGG database annotation of differentially expressed genes indicated that the following genes were related to flavonoid synthesis: phenylpropanoid biosynthesis genes (PAL, C4H, 4CL and CHS), flavonoid biosynthesis genes (E2.1.1.104, CHI, FLS, F3′5′H and ANR), anthocyanin biosynthesis genes (ANS, DFR, HCT, BZ1, GT1, and UGT79B1), isoflavonoid biosynthesis genes (HIDH and CYP81E17), and their transcriptional regulator (MYB, WD40 and bHLH). The types and contents of anthocyanins in the red chestnut were significantly higher than those in the green leaf cultivar ‘Song Jiazao’, especially morning glory 3-O-glucoside, delphinidin 3-O-glucoside, and pelargonium-3-Oglucoside, which were not detected in ’Song Jiazao’. Combined omics analysis showed that downregulated expression of C4H, CHS and F3′5′H and upregulated expression of FLS reduced the supply of raw materials for anthocyanin synthesis, and downstream ANR upregulation converted anthocyanins to procyanidins, increasing the total flavonoid content. F3′5′H was significantly depleted in the leaves and spines of red chestnut compared with ‘Song Jiazao’, while ANS and BZ1 were enriched significantly. It is concluded that C4H, CHS, FLS, F3′5′H, BZ1, ANR and ANS are the key genes needed for breeding red chestnut. This high-quality red Chinese chestnut genome represents an important resource for the chestnut genomics community and transcription and metabolism identified the source of differences and screened out some key differential genes. Those results will improve our understanding of chestnut biology and evolution.

Keywords: *Castanea mollissima*; Red mutant; Genome; Transcriptome and metabolome; Differential genes; Anthocyanin

## Identification of a new QTL for ink disease resistance caused by *Phytophthora cinnamomi* in a chesnut interspecific hybrid progenie

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Abstract

Ink disease caused by *Phytophthora cinnamomi* is one of the most serious diseases affecting chestnut. In recent years a broad range expansion and an increasing severity of ink disease on chestnuts is forecasted with global change. Understanding the control of resistance to *Phytophthora cinnamomi* is essential for the development of new chestnut stand management and research approaches and for the implementation of new breeding strategies. A forward genetic study for this trait was performed using two F1 chestnut interspecific progenies. Resistance to *P. cinnamomi* was measured during three consecutive years. A total of 148 polymorphic SNPs markers, 72 polymorphic SSR markers and 78 polymorphic SSR-seq markers were used to genotyped trees of either or both families (*C. sativa x C. crenata* : 128 individuals; *C. sativa* x *C. mollissima* : 97 individuals). Quantitative trait locus (QTL) analysis allowed the identification of one new genomic region accounting for 37% of the phenotypic variation in one family, stable for the several years tested. These results give new insights of the genetic determinism of resistance to *Phytophthora* and provide new cues for the identification of genes implicated in the control of this trait.

Keywords: Quantitative trait loci (QTL), *Castanea* sp, ink disease, *Phytophtora cinnamomi*

## Establishment of an efficient somatic embryo regeneration system and CmGRF7 modulates the development of somatic embryos in Chinese chestnut

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## Abstract

Chinese chestnut (*Castanea mollissima* Blume), an economic forest species, has important economic value and ecological function. The innovation, improvement, reproduction and utilization of clonal germplasm are the critical foundation for industrial development of Chinese chestnut. Somatic embryogenesis (SE) is an important method to obtain clonal plants for large-scale production. Synchronization rates of somatic embryos at four developmental stages were significantly enhanced by screening via steel sieve after liquid suspension culture. After the selection of mature cotyledonary embryos, the germination rate was increased from 2.2% to 13.3%. We also identified high-embryogenic-competence lines among tested embryogenic cell lines. Furthermore, GRF7 and GIF1 were dramatically highly expressed in the high-embryogenic-competence lines at EC stage, offering effective marker genes to early identify high-embryogenic-potential lines. In protoplasts of Chinese chestnut, CmGRF7 was localized in the nucleus. The number of somatic embryos derived from *CmGRF7*-OE line was significantly larger than the EV line, indicating that overexpression of *CmGRF7* accelerated the development of somatic embryos. The number of somatic embryos derived from *CmGRF7*-RNAi line was significantly smaller than the EV line, demonstrating that down-regulated expression of *CmGRF7* repressed the embryogenic competence. Therefore, *CmGRF7* positively regulates the development of somatic embryos in Chinese chestnut. This study will accelerate large-scale asexual propagation based on somatic embryogenesis of excellent chestnut germplasm for Chinese chestnut provides a fundamental platform.

Keywords: Chinese chestnut; Somatic embryo; Regeneration; CmGRF7