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Comprehension of resistance to diseases in chestnut

Compreensão da resistência a doenças no castanheiro

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ABSTRACT

Ink disease (caused by the pathogen *Phytophthora cinnamomi*) and chestnut blight (caused by the fungus *Cryphonectria parasitica*) are the two most destructive diseases affecting European chestnut (*Castanea sativa*) and American chestnut (*C. dentata*). Therefore, breeding for resistance to both pathogens is essential for the chestnut sustainability in Europe and the United States of America. Several genomic approaches have been implemented in order to map the resistance first to ink disease and then to blight chestnut using a hybrid population: *C. sativa* crossed with resistant Asian species (*C. crenata* and *C. mollissima*). The transcriptome of *C. sativa* and *C. crenata* inoculated and not with *P. cinnamomi* was recently sequenced and allowed the identification of genes putatively involved in ink disease resistance. Taking advantage of molecular markers (microsatellite and SNPs), developed from the referred tanscriptomes and from *C. mollissima* transcriptome, the first *C. sativa* x *C. crenata* genetic map was constructed. Additionally, a reliable phenotyping method was established to evaluate the level of *P. cinnamomi* resistance of each hybrid allowing the identification of two Quantitative Trait Loci (QTLs). These results are the first step for understanding the resistance to ink disease in chestnut.

Keywords: *Castanea* spp., phenotyping, *Phytophthora cinnamomi*, Quantitative Trait Loci, transcriptomics.

RESUMO

A tinta e o cancro do castanheiro, causadas respetivamente pelo oomiceta *Phytophthora cinnamomi* e pelo fungo *Cryphonectria parasitica*, são as doenças que mais severamente afetam o castanheiro europeu (*Castanea sativa*) e americano (*C. dentata*). Assim, o melhoramento para resistência a estes dois agentes patogénicos é essencial para a sustentabilidade do castanheiro na Europa e nos Estados Unidos da América. Diversas abordagens genómicas foram implementadas com o objetivo de mapear a resistência à tinta, e posteriormente ao cancro, usando uma população híbrida: *C. sativa* cruzada com espécies asiáticas resistentes (*C. crenata* e *C. mollissima*). Foi recentemente sequenciado o transcritoma de *C. sativa* e *C. crenata* com ou sem inoculação com *P. cinnamomi*, permitindo a identificação de genes potencialmente envolvidos na resistência à tinta. O primeiro mapa genético de *C. sativa* x *C. crenata* foi construído a partir de marcadores moleculares (microsatélites e SNPs) desenvolvidos a partir daqueles transcritomas bem como do transcritoma de *C. mollissima*. Adicionalmente foi estabelecido um método de fenotipagem para avaliar o nível de resistência de cada híbrido a *P. cinnamomi*, permitindo a identificação de dois QTLs. Estes resultados constituem o primeiro passao para a compreensão da resistência à tinta no castanheiro.

Palavras chave: *Castanea* spp., fenotipagem, *Phytophthora cinnamomi*, QTL, transcritómica.

INTRODUCTION

The genus *Castanea* belongs to Fagaceae, a plant family that dominates much of the climax hardwood forests of the Northern Hemisphere (Manos *et al*., 2008). The European chestnut (*Castanea sativa* Mill.) is considered to be the only native species of *Castanea* in Europe. Chestnuts are multipurpose trees being used in the food industry, for its edible nuts that present high quotation in international markets; in the wood industry, as timber; and also for ecological and landscaping purposes. Taken together, chestnuts have a major economic importance in the Mediterranean region. Chestnut fruit production has declined considerably during the 20th century in southwestern Europe due particularly to the emergence of heavily damaging diseases and pests: ink disease (*Phytophthora* spp.), blight disease (*Cryphonectria parasitica* (Murr.) M.E. Barr) and more recently gall wasp (*Dryocosmus kuriphilus* (Yasumatsu)). In Portugal, a serious decline of chestnut growing area and productivity per hectare has been observed since the last century; the current area is 34,000 ha for fruit production and 41,000 ha for the total area (including forest); *P. cinnamomi* was introduced in 1838, however its negative impact was recorded at the beginning of 20th century, reducing fruit production to 80,000 ha. The actual area is less than half of the country's potential as well as the productivity per hectare. The main threats that contribute for this scenario are the diseases and pests referred above. For chestnut blight and gall wasp the biological control is being used with success in different European countries (the hypovirulence for blight and the spread of the parasitoid *Torymus sinensis* for gall wasp). On the contrary for *Phytophthora* species, there is no known effective option of biological control or chemicals adding the fact that to cope with different hosts and host tissues.

Phytophthora species have evolved sophisticated mechanisms to manipulate plant cells and cause infections. It has been noted that the pathogen grows as a hemibiotroph under certain circumstances with an initial biotrophic and a later necrotrophic stage (Cahill *et al*., 2008) or found to switch between the modes (Shearer and Crane, 2012). Within chestnut genus, Japanese and Chinese chestnut (*C. crenata* and *C. mollissima*, respectively) show resistance to *P. cinnamomi* e *C. parasitica*. In order to contribute to inverting the situation of decline of chestnut stands, a breeding program was initiated in 2006, by this team, aiming at introgressing resistance genes from the resistant Asian species (*C. mollissima* and *C. crenata*) into *C. sativa*, to understand molecular and genetic mechanisms of resistance to ink disease and blight in chestnut and to identify markers linked to resistance to be used for marker assisted selection (MAS).

Portuguese chestnut breeding program

Portuguese chestnut breeding program has been based on interspecific crosses performed between the European chestnut and the Asian species of *Castanea crenata* and *Castanea mollissima*. Two full-sib pedigrees have been produced, SC (*C. sativa* x *C. crenata*) and SM (*C. sativa* x *C. mollissima*) (Costa *et al*., 2011). In order to better understand the molecular and genetic mechanisms we have been integrate two different approaches: mapping and transcriptomic. The scientific main goal of the program is to perform marker: trait association in order to identify Quantitative Trait Loci (QTLs) - mapping approach. To achieve this goal, a common marker framework was used between United States and Portugal for genotyping F1 progenies of SC and SM crosses using microsatellites and SNPs (Kubisiak *et al*., 2013) *P. cinnamomi* resistance levels were evaluated for each individual progeny plant and a set of resistant genotypes were selected (Santos *et al*., 2014). These genotypes were established *in vitro* and are being propagated in large scale. Additionally, a transcriptomic approach was also implemented by sequencing of root transcriptomes of *C. sativa* and *C. crenata* inoculated and not with *P. cinnamomi*. Transcriptome analysis allowed the better elucidation about general chestnut defense mechanisms to ink disease (Serrazina *et al*., 2015). Differential expressed genes in *C. sativa* and *C. crenata* sequences were also used to develop new microsatellite markers to be used in mapping approach.

Phenotyping of *Castanea* hybrids to *P. cinnamomi*

A protocol was established for phenotyping the progenies in terms of resistance/ susceptibility to *P. cinnamomi* using two tests: test 1 - inoculation of roots and test 2 - excised shoot inoculation. In the root inoculation test, 20 genotypes were used: 16 from SC cross and 4 from SM cross. Biological replicates were obtained by *in vitro* clonal propagation. Two plantlets of each genotype were used as control (without inoculation) and an average of 6.85 plantlets were inoculated per genotype. Disease symptoms

were evaluated using 5 different variables: days of survival, root rot level, percentage of root collar rot, percentage of shoot internal lesion and percentage of shoot external lesion. The number of days of survival after root inoculation was the best discriminator of resistance, while the percentage of internal lesion of the longest shoot was the symptom most associated with survival (Santos *et al*., 2014). The seven most resistant genotypes selected from the root inoculation tests were established *in vitro*, for mass propagation.

In what concerns the excised shoot inoculation test, 63 genotypes were tested (45 SC and 18 SM) in two seasons: Spring and Autumn, with 7,9 and 7,6 replicates inoculated respectively in each season. The lesion length was recorded at days 5, 7, 9, 12 and 14 after inoculation with *P. cinnamomi* and lesion progression rate in cm/day was calculated. For the majority of genotypes, the lesion length in the shoots increased over time. In addition, for the most resistant genotypes the lesion length stopped at a given time point, until the end of the experiment (Santos *et al*., 2014).

Analyzing both inoculation tests, the lesion progression rate in the excised shoot inoculation test was strongly and negatively (-0.89) correlated with survival in the root inoculation test. Different responses to *P. cinnamomi* were observed in the progenies: a continuous range of resistance–susceptibility levels among genotypes was observed. The excised shoot inoculation test appears to be a reliable approach for screening the resistance of chestnut genotypes to *P. cinnamomi* (Santos *et al*., 2014).

Phenotyping of *Castanea* hybrids to *C. parasitica*

Phenotyping for blight resistance started when the progenies had woody stems (in the 8th cycle of development) and consisted on the inoculation of 12 genotypes: 6 resistant and 6 susceptible to *P. cinnamomi*. Experiments were performed in February of 2014, when stems were in dormant period. Nine replicates were inoculated per genotype consisting of 3 stems x 3 inoculation points/stem. The measurement of lesion length in cm was recorded for days 10, 14, 17 and 21 after inoculation with *C. parasitica*. Lesion progression length was calculated in cm/day varying from 0,51 cm/day in the most resistant genotype to 1,04 cm/day in the most susceptible genotype. Previous studies also report different responses to *C. parasitica* in *Castanea* hybrids in dormant periods (Bolvanský *et al*., 2014).

A hybrid clone from SM cross was the least affected by *P. cinanmomi* and *C. parasitica* when both biotic stresses were analyzed altogether. This was the first step to evaluate how the different genotypes with different susceptibilities to *P. cinnamomi* respond to *C. parasitica* infection.

Transcriptomic approach

Root transcriptomes of the susceptible species *C. sativa* and the resistant species *C. crenata* were compared in non-inoculation conditions (control) and after *P. cinnamomi* inoculation. Two pools of cDNA libraries were constructed using RNA extracted from roots of C. *sativa*, inoculated and non-inoculated with *P. cinnamomi* and two other pools of cDNA library were made from roots of *C. crenata* at identical conditions. Each pool included roots of three replicates (3 plants of the same genotype collected at 3 time points (48 h, 96 h and 7 days after inoculation)). The libraries were sequenced using 454 sequencing platform. Pyrosequencing produced 771,030 reads and assembly set up 15,683 *contigs* for *C. sativa* and 16,828 for *C. crenata*. Gene Ontology annotation revealed terms related to stress as "response to stimulus", "transcription factor activity" or "signaling" for both transcriptomes. Differential gene expression analysis revealed that *C. crenata* involved more genes related with biotic stress upon pathogen inoculation than *C. sativa*. Those genes are involved for both species in the regulation of plant immune response and stress adaptation and recovery. Furthermore, it is suggested that both species recognize the pathogen attack; however, the resistant species may involve more genes in the defense response than the susceptible species. RNA-seq enabled the selection of candidate genes for ink disease resistance in *Castanea* (Serrazina *et al*., 2015).

Utilizing this transcriptome data, we developed 43 new microsatellite markers from the gene differentially expressed sequences in European chestnut (*Castanea sativa*) and Japanese chestnut (*Castanea crenata*) in response to inoculation with the pathogen. Twenty-four parent and progeny trees – representing *P. cinnamomi* susceptible, European and American chestnut (*C. dentata*), and *P. cinnamomi* resistant, Japanese and Chinese chestnut species and some of their inter-species hybrids –were used to evaluate the microsatellite markers' polymorphism and transferability rates within and among species,

respectively. The set microsatellite markers showed a remarkably high interspecific transferability rate among the four *Castanea* species tested (*C. sativa*, *C. crenata*, *C. dentata* and *C. mollissima*), ranging from 90.7% for Chinese chestnut and 100% for European chestnut. Only three microsatellite markers were monomorphic (7%) and the average value of expected heterozygosity was 0.61, higher than that in other studies using microsatellite developed in transcriptomes in chestnut (Santos *et al*., 2015).

The novel microsatellite markers developed and characterized are useful for constructing genetic linkage maps, conducting QTL analyses of phenotypic traits, genotype–phenotype association studies (especially in relation of resistance *to P. cinnamomi*), high-throughput genotyping for clonal identification or marker-assisted selection, and comparative genomics between the genetic linkage maps generated by the American chestnut breeding team and our team.

Selection and propagation of hybrid chestnut genotypes resistant to *P. cinnamomi*

Genetic linkage maps are being produced for identifying areas within the genome linked to resistance, to identify molecular markers that allow for early selection of resistant genotypes, from the current breeding program. Currently, the selection of clones is done by inoculating the roots with the pathogen, which is a time-consuming process. When these markers are available we will be able to know, with a simple DNA extraction, which are the most resistant genotypes. The most resistant genotypes, selected so far from the breeding program, are being mass propagated by micropropagation (Figures 1 and 2) and tested in field conditions, in different edaphoclimatic conditions and also in terms of graft compatibility with Portuguese varieties for fruit production, following the protocol of Pina *et al*. (2012). The goal is to release into the industry in the next five years, new improved plant material to boost the productive chestnut sector, since both Portugal and Europe have a high deficit of improved material for plantation. In the near future we will be able to provide a wide range of rootstocks with genetic variability, adapted to different conditions of soil and climate. The idea is to produce a catalog of different genotypes with pedigree and genetic identity, determined by molecular markers, containing information on which rootstocks is better adapted to each region and what the variety has better compatibility for each rootstock.

Figure 1 - Micropropagation of chestnut hybrids resistant to ink disease.

Figure 2 - Chestnut hybrids acclimatization in the greenhouse.

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