Differential Gene Expression in Chestnut Buds Following Infestation by Gall-Wasp (*Dryocosmus kuriphilus* Yasumatsu, Hymenoptera: Cynipidae)

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Abstract

The chestnut gall-wasp *Dryocosmus kuriphilus* Yasumatsu, an insect native of China, was found in 2002 for the first time in Europe, and specifically in North western Italy (Cuneo province). The cynipid lays eggs into dormant buds and, in the following year, causes the development of galls on leaves, sprouts and inflorescences, due to the feeding activity of larvae. The wasp has an effect both on growth and yield of the tree. Complete resistance to *D. kuriphilus* was recently found in the hybrid Bouche de Bètizac (*C. sativa* × *C. crenata*) and the genetic bases regulating this resistance are being investigated. Accessions of the cultivars Marrone (susceptible) and Bouche de Bétizac (resistant) were subjected to an intensive attack of *D. kuriphilus*, with the purpose of getting infested buds to be examined at different stages of development of larvae. At budburst and just afterwards (March-April) buds were collected for the extraction and isolation of mRNA. cDNA was obtained by reverse transcription and analysed using the differential display technique. Two protocols, differing for the primer design, were applied and the results showed that 13 mer primers in combination with one-base anchored oligo-dT primers produced the best scorable profiles. Differentially expressed bands were cloned, sequenced and blasted with sequences deposited in NCBI and TIGR databases; putative genes were identified. The results showed that early activity of larvae in susceptible genotypes particularly influences the expression of genes involved in differentiation and nourishing; other genes found differentially expressed in infested tissues are involved in the plant response to external stimuli.

INTRODUCTION

In 2002 the presence of the insect *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae) was reported in chestnut (*Castanea sativa* L.) groves and forests of Cuneo Province (Piedmont region, Italy) (Brussino et al., 2002). The wasp is univoltine and thelytokous and spreads locally mainly by active flight of adults, sometimes favoured by winds. On long distance it occurs with the commercialisation of propagation material and accidental transport of adults due to the flow of people. It lays eggs into dormant buds and, in the following year, larvae induce the formation of galls on the developing organs (leaves, floral organs, whole sprouts) compromising their growth. Plant production (nuts, timber) can be drastically reduced when the infestation is high.

Although biological control is thought to be the most appropriate solution to reduce the infestation in forest areas, the maximum level of containment of this gall wasp in orchards will be obtained by using chestnut selections bearing resistance or reduced susceptibility to the cynipid. Several cultivars, prevalently belonging to the species *Castanea crenata* and its hybrids, are considered resistant; among them, the hybrid Bouche de Bétizac (*C. sativa* × *C. crenata*) was recently reported.

In this paper differential gene expression was studied in a susceptible and in a

resistant cultivar, in order to understand the molecular mechanisms of response to the presence of the larvae in the bud.

The differential display technique used in this research was developed in 1992 by Liang and Pardee, with the purpose to study in rapid, sensitive and accurate way the alteration of the genetic expression in the carcinogenic cells. The basic principle of differential display technique is to amplify the cDNA obtained from mRNA extracted from 2 tissues/organs which are maintained in the same conditions except for one single factor that is supposed to make a difference in term of gene expressions. In this case, infested and not infested organs of 2 cultivars with different response to gall wasp (resistant vs susceptible) were compared. Preliminary results are presented and the methodological approach is discussed.

MATERIAL AND METHODS

In 2005 and 2006 summers, accessions of the cultivars Marrone (*C. sativa*) and Bouche de Bétizac were subjected to an intensive attack of *D. kuriphilus*, with the purpose of getting infested buds to be examined at different stages of development of the larvae. Although Bouche de Bétizac is resistant, the wasp is able to lay eggs into its buds but larvae do not survive beyond the first instar.

At budburst and just afterwards (March-April) buds were collected both from the infested plant and from not infested ones, used as control; samples were immediately frozen in liquid nitrogen. Buds were ground and prepared for the extraction of the nucleic acids according to the protocol by Chang et al. (1993), with some modifications. The total RNA was purified by the RNeasy® Plant mini Kit (Qiagen, Germany) and then the cDNA was synthesized using SuperscriptTMII (Invitrogen™, USA) and the oligo-dTs afterwards described.

At a first stage, the differential display technique was carried out comparing the gene expression in infested buds of Marrone and of Bouche de Bétizac (2 theses) and performing PCR with two base-anchored oligo-dT ($(T)_{12}$MA/MC/MG) primers combined with decamer primers (Operon Tech., USA). At a second stage a 3rd thesis "not infested Bouche de Bétizac" was introduced and PCR was carried out using one-base anchored oligo-dT ($(T)_{13}$A/C/G) primers in combination with 13 mer primers (RNA image Kits®, GenHunter, USA).

In the first case the PCR mix (20 µl) included 3 mM $MgCl_2$, 500 µM dNTPs, 0.1 pmol/µl of each primer, 1 U of Taq polymerase (Bioline, Italy) and 2 µl of template cDNA; amplification was done with the following conditions: one cycle of 2' at 95°C, 5' at 40°C and 2' at 72°C followed by 40 cycles of 30'' at 95°C, 1' at 40°C, 2' at 72°C and by a final extension step of 7' at 72°C.

The PCR of the combination between one-base anchored oligodT and 13 mer primers was performed in a final volume of 20 µl (3 mM $MgCl_2$, 20 µM dNTPs, 0.2 pmol/µl primers) using 1 U of Taq polymerase (Bioline, Italy) and 2 µl of template cDNA with the following steps: 30'' at 94°C, 2' at 40°C, 30'' at 72°C (40 cycles) followed by one final elongation step of 5' at 72°C. Amplicons were separated by electrophoresis on polyacrilamide gel (5%) and stained with silver nitrate (Caetano-Annoles and Gresshoff 1994, with some modifications). Differentially expressed bands were extracted from the gel, according to the protocol by Basak et al. (2006), were riamplified and cloned in *E. coli* after ligation in a plasmid vector (Kit Topo TA Cloning®, Invitrogen™, USA). Differentially expressed fragment were sequenced by capillary electrophoresis on ABI 3130 Genetic Analyzer (Applied Biosystems); they were blasted with sequences deposited in TIGR (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/Blast/index.cgi) and NCBI databases (www.ncbi.nlm.nih.gov/blast/).

RESULT AND DISCUSSION

The transcriptome in a cell can be investigated through different techniques, each with the final purpose to compare the mRNA expression among the studied samples. The choice in the application of the different methods of investigation depends on the type of

study that is wanted to effect and from the degree of resolution that each of these offers.

In the Differential Display technique (Liang and Pardee, 1992) a limited number of short arbitrary primers is used in combination with anchored oligo-dT primers to systematically amplify and visualize most of the mRNA in a cell. The optimal length of arbitrary primers is determined by statistical consideration that each primer will recognize 50-100 mRNA species. To do so, these primers have to hybridize as 6-8 mers (Liang and Pardee 1992). In practice, however, primers shorter than 9 bases failed to be used for PCR amplification (Williams et al., 1990) probably due to the minimum contact surface between Taq polymerase with the double-stranded DNA template. According to Liang et al. (1994), 13mers are the optimal arbitrary primers but in many researches also decamer primers are still used.

As regards the anchored oligo-dT primers, the initial choice of using two-base anchored oligo-dT primers (Liang and Pardee, 1992) instead of one-base anchored primers (Liang et al., 1994) was due to the failed attempt to amplify the 3' terminus of some sequences in combination with an upstream primer specific. Later, longer one-base anchored primers were shown to be much more efficient for differential display in sub-dividing the mRNA populations into three groups.

In the present work decamer and two-base anchored oligo-dT primers were initially tested in 99 combinations. Good and reliable electrophoretic profiles were obtained for 16 combinations and 44 differentially expressed fragments were selected; 21 of them were present only in Marrone and 23 only in Bouche de Bètizac. 34 of these were successfully riamplified and 30 ligated and cloned.

Later, forty eight 13 mer primers were used in combination with 3 different one-base anchored oligo-dT. Good and reliable electrophoretic profiles were obtained from 108 combinations and 77 differentially expressed fragments were selected; 75 of them were present only in Marrone and 2 only in Bouche de Bètizac. Forty five of these were successfully riamplified and 42 ligated and cloned.

The profiles obtained using the 13 mer primers (Fig. 1) were of better quality in comparison with those obtained with the decamer ones, confirming data reported in literature (Liang et al., 1994). In particular, the number of combinations with scorable profiles was only 16% for decamer primers paired with the two-base anchored oligo-dT primers, and the improved resolution obtained with 13 mer primers allowed a better band scoring and excision and finally a more accurate sequencing.

In addition, according to literature, the use of one-base anchored primers have significant advantages over the use of two-base anchored primers: the former reduces the redundancy of priming, eliminates the high background and the smearing problem for two-base anchored primers ending with the 3' "T", and reduces the number of reverse transcription reactions from 12 to 3 per RNA sample (GenHunter Corporation: http://www.genhunter.com).

The introduction of the 3rd thesis (not infested buds of Bouche de Bétizac), analysed only with the GenHunter kits, greatly reduced the number of differentially expressed bands found in the infested Bouche de Bétizac buds.

The alignment of the sequences found with those in databanks through the program BLAST allowed to draw preliminary hypotheses about the molecular bases of the plant response to *Dryocosmus kuriphilus*. The recovery of sequences putatively coding for resistance genes, mitogen activated proteins, vesicle-associated membrane proteins and 14-3-3 proteins may indicate the existance of a hypersensitive response in Bouche de Bétizac and its inhibition in Marrone. Concerning gall formation and larva nourishment, the isolation of sequences putatively coding for lipid-associated proteins and carrier proteins involved in the lipidic metabolism provided confirmation to the theory by Schönrogge et al. (2000); in fact, nutritive tissues of cynipid galls are know to contain large numbers of liposomes and generally high amounts of glycerid lipid.

These preliminary results require confirmation, first of all testing the expression of the putatively identified genes in infested and not infested tissues of the susceptible and resistant genotype using Northern Blot and Real Time PCR. In addition, further

differential display analyses will be carried out with more 13 mer primers combined with one-base anchored oligo-dT primers, in order to cover at least 90% of the transcriptome.

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Figures

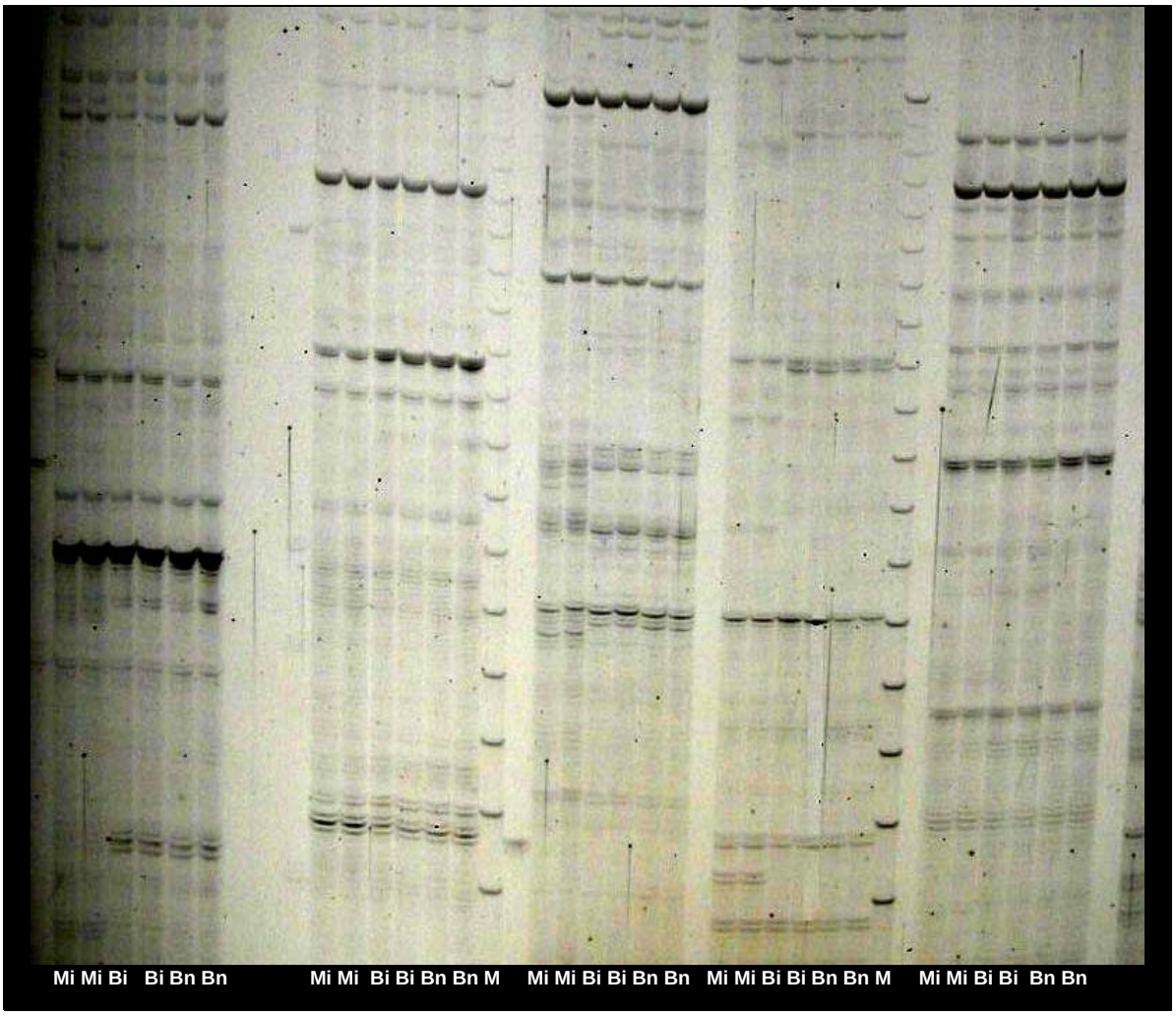

Fig. 1. Electrophoretic profiles obtained from the DD amplification using one-base anchored oligo-dT primers in combination with 13 mer primers. Each primer combination was used to amplify in duplicate the cDNAs from the 3 theses (Mi= infested Marrone; Bi=infested Bouche de Bétizac; Bn=not infested Bouche de Bétizac; M=10-bp DNA ladder, Invitrogen^{TM}, USA).