Physiological and Molecular Plant Pathology 92 (2015) 1–13

Contents lists available at ScienceDirect

Physiological and Molecular Plant Pathology

journal homepage: www.elsevier.com/locate/pmpp

Identification of *Theobroma cacao* genes differentially expressed during *Phytophthora megakarya* infection

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ARTICLE INFO

Article history:
Received 18 February 2015
Received in revised form
27 July 2015
Accepted 10 August 2015
Available online 15 August 2015

Keywords:
Differentially expressed genes
Macroarray screening
Phytophothora/plant interaction
Protease inhibitor
Real-time PCR
Suppression subtractive hybridization (SSH)

ABSTRACT

Cocoa black pod caused by *Phytophthora* is one of the most serious diseases affecting cocoa production. This project set out was to improve our knowledge of the molecular mechanisms involved in *Theobroma cacao* resistance. We performed a transcriptional screening using macroarrays containing 89 ESTs from subtractive libraries enriched with genes differentially expressed in response to *T. cacao*/*Phytophthora* interactions. A gene expression analysis revealed the induction of genes involved in stress signal transduction, general and specific defence genes and genes involved in hormone signalling pathways. RT-qPCR showed that the level expression of two genes in the resistant plant was higher during the first eight hours after pathogen inoculation. Three genes from a family encoding a protease inhibitor displayed different levels of expression depending on resistant and susceptible *T. cacao* clones. A comparison of promoter sequences revealed a motif present only in genes that were up-expressed in the resistant plant.

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1. Introduction

Cocoa black pod caused by several species of *Phytophthora* is one of the most serious diseases affecting cocoa (*Theobroma cacao* L) production [1]. *Phytophthora capsici* can cause up to 10% yield losses while *Phytophthora palmivora* can be responsible for 30%. Infection by *Phytophthora megakarya* can result in up to 80% of yield losses [2]. *P. megakarya*, is therefore the most aggressive species on cocoa and is endemic to Africa. *P. palmivora* is present on all the continents where cocoa is produced, and *P. capsici* only in America. These two species can attack many other tropical plants, whereas the only known host of *P. megakarya* is cocoa.

Phytophthora are members of the Stramenopiles order, Oomycetes group and Peronosporaceae family [3]. The genomic sequencing of 3 species of *Phytophthora* (*Phytophthora sojae*, *Phytophthora ramorum* and *Phytophthora infestans*) opened up the way for understanding the molecular mechanisms involved in the virulence of each of these pathogens [4]. Genomic data has provided a clearer understanding of the evolutionary history of the genus and made it possible to adjust the clade number of the *Phytophthora* phylogenetic tree. In fact *P. megakarya* and *P. palmivora* are included in clade 4, while *P. capsici* is found in clade 2 [5].

P. megakarya is currently taking over from *P. palmivora* in Africa, the leading cocoa producing continent (between 45 and 55% of world production). This expansion could have a very bad impact on the cocoa market. Chemical control of this disease is laborious, expensive and the genetic improvement of planting material appears to be an efficient alternative [1].

Until now, cocoa resistance to *Phytophthora* sp has been described as a "race-nonspecific like" type and, in all the germplasm analysed, no tree has ever been found to be completely resistant in the field [6]. Studies on the genetic control of cocoa resistance to three species of *Phytophthora* (*P. palmivora*, *P. megakarya* and *P. capsici*) have shown QTLs located in many genomic regions [7–11], with some of them common to the three *Phytophthora* species. This situation offers the possibility of improving resistance in cocoa by a possible accumulation of many

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http://dx.doi.org/10.1016/j.pmpp.2015.08.005
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different resistance genes located in different chromosome regions using marker-assisted selection.

Genetic resistance is classed as qualitative resistance (mediated by resistant genes (R genes)) and as quantitative resistance (controlled by many interacting genes that slow down the development of the pathogen [12].

Several R genes of *Solanum tuberosum* and *Glycine max* conferring resistance to *P. infestans* and *P. sojae*, respectively, have been characterized [13]. These R gene classes encode proteins with conserved domains such as nucleotide-binding sites (NBS) and C-terminal leucine rich repeats (LRR). This NBS-LRR structure is common to R genes conferring resistance to oomycetes. It differs from their N-terminal which should be a leucine zipper domain (LZ) or a Toll/interleukin-1-receptor homology region (TIR). The potato R1 gene belonging to the LZ/NBS/LRR class has been cloned and its functionality has been verified in transgenic tomato [14].

The resistance conferred by R genes is very efficient, but also specific to a particular strain and might be avoided by a mutant or a new strain. Hence, qualitative resistance is less durable than quantitative resistance. Indeed, the latter is usually indicated as multi-genic, hence more difficult for the pathogen to overcome.

In addition to R genes, plants develop downstream inducible defence responses, such as pathogenesis-related proteins (PRs), secondary metabolism, lignification, etc. [15]. The induction of potato defence gene transcripts has been reported during *P. infestans* infection [16]. Those genes encode chitinase (PR-3, 8 and 11), osmotin (PR-5), peroxidase (PR-9), cytochrome P450, or transcription factors. In avocado plants, infection by *Phytophthora cinnamomi* induces proteinase inhibitors (PR-6), which block the pathogen's extracellular proteases [17].

The molecular basis of *Phytophthora megakaria/T. cacao* resistance is poorly understood. Among the methods of analysis, those concerning differentially expressed genes might contribute to a better understanding of the molecular processes involved in this pathosystem. More than 300 ESTs corresponding to genes induced during the defence response have been isolated from *T. cacao* leaves treated with defence response elicitors [18]. Sequences are distributed in the major functional classes, such as cell division, defence (chitinase-like protein, heat shock protein 70, dnaK-type molecular chaperone, etc.), signal transduction, cellular organization, cellular transport and metabolism. Among them, a few genes have already been described as being enhanced or repressed during susceptible interactions between *T. cacao* leaves and *P. megakarya* [19]. This differential expression is also influenced by leaf developmental stages, suggesting that defence mechanisms differ during leaf development.

The aim of this work was to identify candidate genes involved in *T. cacao* resistance to *P. megakarya* using functional genomic approaches. Possible involvement of the identified genes in the cocoa response to this pathogen is discussed.

2. Materials and methods

2.1. Plant material

The study was conducted on 2 clones from a progeny of 345 individuals created in Papua New Guinea and derived from the cross of parent 17-3/1 with parent 36-3/1 [11].

These parents have genetic origins resulting from Trinitario and Upper Amazon Forastero which provide flavours and robustness/productivity respectively. Parent 17-3/1 carries resistance to *Phytophthora* spp [20]. Clone 286 (PNG-R) was chosen for its resistance response and on its most favourable allele combination. The clone 156 (PNG-S) was chosen based on its susceptible response and its less favourable allele combination. The two tree clones, PNG-R and PNG-S, have been cultivated in Montpellier for more than eight years in a greenhouse and were cut to produce branches to collect enough leaves for the experiment that were at the same stage from non-lignified twigs.

2.2. Inoculation experiments

The leaf inoculation procedure was described by Nyassé et al. 1995 [21]. *P. megakarya* strain NS 269 described for its high level of pathogenicity [1] was used in the experiments in order to have strongly contrasted disease symptoms between the PNG-R and PNG-S clones.

Ten whole leaves at the same stage from non-lignified twigs were collected from PNG-R and PNG-S trees. Ten microlitres of a suspension containing $3 \cdot 10^5$ zoospores mL^{-1} was applied to the abaxial surfaces of excised leaves. Controls were treated with sterile distilled water droplets. Each experiment consisted of twenty droplets of inoculum, ten per longitudinal half of the leaves. One longitudinal section was used for RNA extraction. Leaf discs one cm across around the inoculum deposits were harvested at various time intervals (0, 2, 4, 8, 24, 48, 72 and 96 h post-inoculation, hpi), pooled by time, immediately frozen in liquid nitrogen and stored at −80 °C.

The second longitudinal section was stored in room culture for 6 days for symptom observations. Disease symptom scoring was based on a scale of 0–5 [21] as follows: (0) no symptoms, (1) necrotic points, (2) connected necrotic points, (3) reticulated lesions, (4) marbled lesions and (5) true necrotic lesions.

Two biological replications were carried out on the same trees 3 months apart in order to collect sufficient leaves from one tree per clone. The samples of the first experiment were used to construct cDNA libraries and test the candidate genes by Rt-qPCR. The samples of the second experiment were used to test the cDNA library by macro-arrays and on the candidate genes by Rt-qPCR.

2.3. RNA isolation and cDNA synthesis

RNAs from each of the pooled samples at each time interval (0, 2, 4, 8, 24, 48, 72 and 96 h post-inoculation, hpi), for each clone (PNG-R and PNG-S) and each experiment were extracted independently.

Total RNA was isolated from up to 100 mg of leaf discs using the TE3D (Tris-EDTA-3 Detergents) method [22]. After extraction, DNase was used to eliminate genomic DNA. The total RNA was re-suspended in 100 μL of water. One hundred ng of total RNAs was reverse-transcribed and amplified using the SMART PCR cDNA Synthesis kit (Clontech, Palo Alto, CA) according to the user's manual.

2.4. Suppression subtractive hybridization, cDNA library construction

The double-stranded cDNA fractions were digested with Rsa I to obtain shorter, blunt-ended molecules, then purified with the QIA quick PCR purification kit (QIAgene). SSH was performed with the PCR-Select cDNA subtraction kit (Clontech), following the manufacturer's instructions.

The experiment used cDNA fragments generated from the mRNA of control and infected tissues from susceptible and resistant individuals, as driver and tester, respectively. The SSH library enriched for differentially expressed cDNAs was constructed by insertion of the subtracted cDNAs into pGEM-T easy vector (Promega, France) and transformed into *Escherichia coli* electrocompetent cells (DH10B101). White colonies were randomly taken from the plated subtractive cDNA libraries and grown in 1 mL of LB-Amp medium in 96-well plates at 37 °C.

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