Use of Leaf-Disk Technique for Gene Expression Analysis of the Coffee Responses to *Hemileia vastatrix* Infection

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SUMMARY

The most acknowledged method for coffee leaf-rust resistance evaluation uses leaf disks inoculated with *Hemileia vastatrix* and kept in moisture chambers. Besides an efficient control of inoculation conditions, this technique allows a simultaneous evaluation of innumerous plants, with diverse fungal race/coffee genotype combinations, and using low uredospore quantities. The objective of this study was to evaluate the suitability of this technique for the functional gene analysis of coffee responses to leaf-rust infection. A comparison of gene expression in the presence or absence of the pathogen was performed on intact leaves and on leaf disks. To avoid non-specific gene expression due to leaf injury, the leaf disks were prepared 24h and 48h before inoculation and kept moist. *Coffea arabica* plant samples of the resistant Obatã and the susceptible Ouro Verde cultivars were challenged with *H. vastatrix* race II and were collected 24 h after inoculation. Semi-quantitative reverse transcription (RT)-PCR and real time quantitative PCR were used to evaluate expression of several coffee genes. Genes known to be constitutively expressed such as the Glyceraldehyde 3-phosphate deshydrogenase gene or the Ubiquitine gene were used, as well as genes involved in disease resistance responses. Results demonstrated that overall there are differences in the gene expression patterns observed in leaves and disks, either prepared 24h or 48h before inoculation. The genes *PAD3* and *PR1b* showed induction in leaves and in the 48h-disks of Obatã upon rust fungus inoculation, and gene suppression in the 24h-disks treatment. The genes *WRKYs* were activated in leaves and suppressed in disks in the same cultivar. Opposite patterns of WRKY expression were detected in disks of Ouro Verde. Our results showed that most of the defense-related genes studied displayed altered patterns of gene expression compared to intact leaves. These results suggest that the leaf-disk technique cannot be successfully used for transcriptomic analysis of coffee-rust interactions.

INTRODUCTION

The orange rust (Hemileia vastatrix Berk. and Br.) is the most important fungal disease of coffee (*Coffea arabica*) in Brazil, and, depending on the defoliation intensity, yield loss, may account for 30% losses in coffee production (Kushalappa and Eskes, 1989).

The Instituto Agronômico de Campinas (IAC), São Paulo, Brazil, develops since the 70's, a genetic breeding program aiming at the development of resistant varieties to the rust fungus. In this program were generated the main resistant coffee cultivars of Brazil, as Icatu Vermelho, Icatu Amarelo, Icatu Precose, Tupi and Obatã.

Despite the high degree of resistance showed for some of these varieties, the fast development of new races of the fungus become the selection of resistant plants a non-stop work.

The most acknowledged method for coffee leaf-rust resistance evaluation uses leaf discs inoculated with *Hemileia vastatrix* and kept in moisture chambers (Eskes and Toma-Braghini, 1981). Besides an efficient control of inoculation conditions, this technique allows a simultaneous evaluation of innumerous plants, with diverse fungal race/coffee genotype combinations, and using low uredospore quantities. The objective of this study was to evaluate the suitability of this technique for the functional gene analysis of coffee responses to leaf-rust infection.

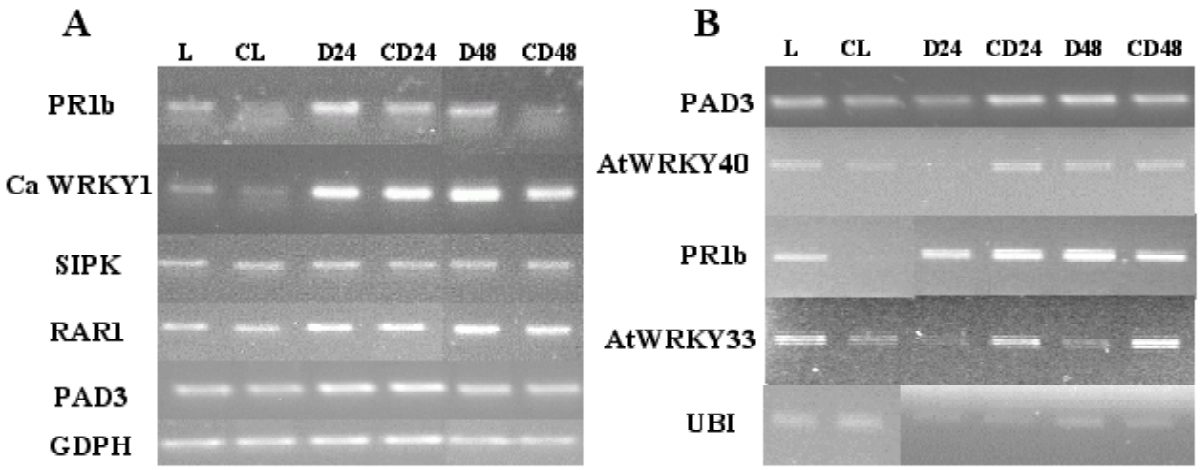
MATERIAL AND METHODS

A comparison of gene expression in the presence or absence of the pathogen was performed on intact leaves and on leaf disks. To avoid non-specific gene expression due to leaf injury, the leaf disks were prepared 24 h and 48 h before inoculation and kept moist. *Coffea arabica* plant samples of the resistant Obatã and the susceptible Ouro Verde cultivars were challenged with *H. vastatrix* race II and were collected 24 h after inoculation. Four eight-month-old coffee plants of each cultivar were kept under a regime of 16h light and 8h dark at 23 ± 2 °C during the assays. Two leaves per plant were inoculated *in situ* and the two opposites leaves were detached for the confection of the disks (1.8 cm diam.), with a cork borer. Each treatment was composed of eight disks. The leaves and disks were inoculated with droplets of 0.025ml spore suspension, with 1 mg spore/ml of distilated water.

Semi-quantitative and quantitative "real time" reverse transcription (RT)-PCR (Ganesh et al., 2006), was used to evaluate expression of several coffee genes previously isolated (Fernandez et al., 2004; Lecouls et al., 2006). Genes known to be constitutively expressed such as the *Glyceraldehyde 3-phosphate deshydrogenase* (*GDPH*) gene or the *Ubiquitine* (*UBI*) gene were used, as well as genes involved in disease resistance responses. The genes chosen for the analyses were: the WRKY transcription factors (*CaWRKY1*, *AtWRKY33*, *AtWRKY40*), bZIP transcription factor (*RAR1*), acid and basic pathogenesis-related gene (*PR1a* and *PR1b*), the salicylic acid induced protein kinase (*SIPK*), non-expressor of PR1 (*NPR1*) and the Cytochrome P450 (*PAD3*).

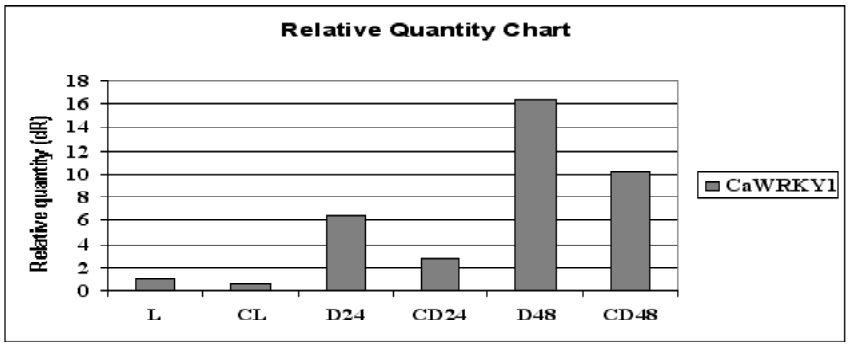
RESULTS

In the semi quantitative RT-PCR analyses, the susceptible cultivar Ouro Verde showed similar patterns of expression between leaves and disks (Figure 1A). The *CaWRKY1* and the *PR1b* were induced in inoculated samples as compared to control samples. Gene expression was slighly higher in inoculated disks than in inoculated leaves, but not in the control disks compared to the control leaves. The inoculation with *H. vastatrix* did not induce the genes *SIPK*, *RAR1* and *PAD3* in these treatments**.** However, in the resistant cultivar Obatã, there was a difference in the gene expression patterns observed in leaves and discs, either prepared 24 h or 48 h before inoculation (Figure 1B). The genes *PAD3* and *PR1b* showed induction in leaves and in the 48h-disks upon rust fungus inoculation. In the 24 h-disks, gene suppression occurred. The genes *WRKYs* were activated in leaves and suppressed in disks. The genes *SIPK* and *RAR1* were not induced and showed similar patterns of expression between leaves and disks in Obatã (data not shown).



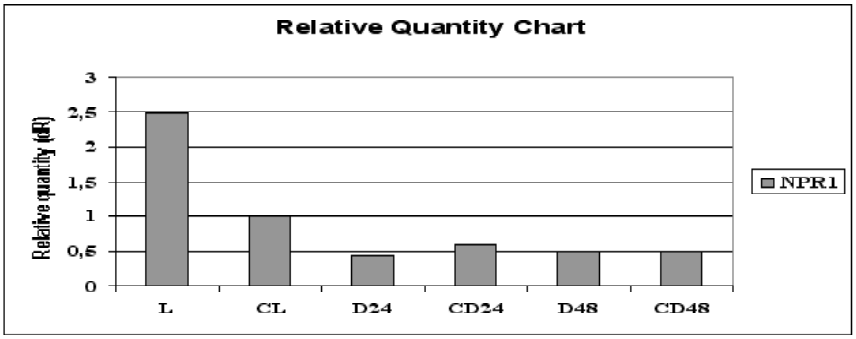
L = inoculated leaves; CL = non inoculated leaves; D24 = 24h-discs inoculated; CD24 = 24h-discs non inoculated; D48 = 48h-discs inoculated; D48 = 48h-discs non inoculated.

Figure 1. Expression of several genes in leaves and leaf discs of two *Coffea arabica* varieties, inoculated with race II of *Hemileia vastatrix.* (A) susceptible variety of coffee Ouro Verde; (B) resistant variety of coffee Obatã.



L = inoculated leaves; CL = non inoculated leaves; D24 = 24h-discs inoculated; CD24 = 24h-discs non inoculated; D48 = 48h-discs inoculated; D48 = 48h-discs non inoculated.

Figure 2. Relative expression of the gene *CaWRKY1* in leaves and leaf disks of the resistant cultivar Obatã of *Coffea arabica*, 24 hours upon orange rust fungus inoculation.



L = inoculated leaves; CL = non inoculated leaves; D24 = discs with 24h inoculated; CD24 = discs with 24h non inoculated; D48 = discs with 48h inoculated; D48 = discs with 48h non inoculated.

Figure 3. Relative expression of the gene *NPR1* in leaves and leaf disks of the resistant cultivar Obatã of *Coffea arabica*, 24 hours upon orange rust fungus inoculation.

In the Real Time RT-PCR analyses, the acid pathogenesis-related gene (*PR1a*), showed similar patterns of expression in leaves and disks treatments (data not shown). In the same way, the gene CaWRKY1 was suppressed in leaves and disks after inoculation in the resistant cultivar Obatã (Figure 2). The *NPR1* gene was quite suppressed in the discs treatments of the Obatã (Figure 3).

DISCUSSION

Our results showed that the stress provoked by the leaf disk cuttings altered the expression of many genes. When challenged with the rust fungus *H. vastatrix*, most of the defense-related genes studied displayed altered patterns of gene expression in coffee leaf discs compared to intact leaves.

Several genes involved in plant pathogen defence mechanisms are also activated by wounding (Cheong et al., 2002). The WRKYs transcription factors are a family of genes activated by many biotic and abiotic stresses. There are strong evidences that the WRKY genes are involved in defence genes regulation, acting upstream genes like PR1 and NPR1 (Yu et al., 2001; Liu et al., 2005). Increased expression of NPR1 and PR1 are related to enhanced levels of resistance (Liu et al., 2005). Here we found that the expression of the WRKYs is hardly affected by the disk lesion and NPR1 was quite suppressed by the disk treatment.

These results suggest that the leaf-disc technique cannot be successfully used for transcriptomic analysis of coffee-rust interactions.

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