IDENTIFICATION AND QUANTIFICATION OF DIFFERENTIALLY EXPRESSED GENES ASSOCIATED WITH CITRUS BLIGHT (*Citrus* spp.)

Identificação e quantificação de genes diferencialmente expressos associados ao declínio dos citros (*Citrus* spp.)

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ABSTRACT

Brazil is the largest citrus producer in the world, being responsible for more than 20% of its production, which is, however still low due to phytosanitary issues such as citrus blight. Citrus blight is an anomaly whose causes still have not yet been determined, therefore there are no efficient control measures to minimize the production losses with the use of resistant varieties being considered the most appropriate method. However, little is known about the genes involved in the defense response of the plants to this anomaly. Considering that many physiological alterations associated with plant stress responses are controlled at a transcriptional level, in this study we sought the identification and characterization of the gene expression products differentially expressed in the response to the citrus blight. Through the suppressive subtractive hybridization technique, expressed cDNA libraries were built using mRNAs isolated from "Cravo" lemon tree roots (*Citrus limonia* L. Osbeck) under "Pera" orange (*Citrus sinensis* L. Osbeck) of healthy and sick plants. 129 clones were obtained by subtraction and their sequences were compared in databases. 34 of them linked to proteins associated to stress processes, while the others were similar to sequences of unknown functions or did not present similarity with sequences deposited in the databases. 3 genes were selected and their expressions were studied by RT - qPCR in real-time. Plants with citrus blight presented an increase of the expression level in two of those genes, suggesting that these can be directly involved with this anomaly.

Index terms: Differential gene expression, subtractive hybridization, RT-qPCR.

RESUMO

O Brasil é o maior produtor de citros do mundo, sendo responsável por mais de 20% de sua produção. No entanto, a produção ainda é baixa, em decorrência de problemas fitossanitários, como o Declínio do Citros que é uma anomalia cuja causa ainda não foi determinada e, consequentemente, não existem medidas de controle para minimizar as perdas na produção. O uso de variedades resistentes é considerado como a medida de controle mais adequada. Contudo, pouco se conhece sobre os genes envolvidos na resposta de defesa das plantas a essa anomalia. Considerando que muitas alterações fisiológicas associadas com respostas a estresses em plantas são controladas em nível transcripcional, neste estudo objetivou-se a identificação e caracterização dos produtos de expressão gênica diferencialmente expressos na resposta ao Declínio dos Citros. Por meio da técnica de hibridação subtrativa supressiva, bibliotecas de cDNAs expressos foram construídas utilizando mRNAs isolados de raízes de limoeiro "Cravo" (*Citrus limonia* L. Osbeck) sob laranja "Pera" (*Citrus sinensis* L. Osbeck) de plantas sadias e doentes. Cento e vinte e nove clones foram obtidos por subtração e suas sequências foram comparadas em bancos de dados. Trinta e quatro delas relacionaram-se a proteínas associadas a processos de estresses, enquanto as outras foram similares a sequências de funções desconhecidas ou não apresentaram similaridade com sequências depositadas nos bancos de dados. Três genes foram selecionados e suas expressões foram estudadas por RT- qPCR em tempo real. Plantas com Declínio dos Citros apresentaram um aumento no nível de expressão em dois desses genes, sugerindo que estes podem estar diretamente envolvidos com essa anomalia.

Termos para indexação: Expressão gênica diferencial, hibridação subtrativa, RT-qPCR.

INTRODUCTION

The citrus blight is an alteration in the normal development of the plant, characterized by accentuated loss of leaves, excess branches and shoots on the trunk, gradual drying of branches, unseasonal flowering, accentuated nutrient deficiencies, accumulation of soluble phenols in the stem, presence of amorphous and/or filamentous obstructions in the xylem vessels and low production (Baldassari; Goes; Tannuri, 2003; Auler et

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al., 2011). The affected plants rarely die, but they become economically unviable due to low amount and quality of the produced fruits. Although this anomaly has been known for more than a century, the causal agent is still not known (Barrios et al., 2006; Brlansky et al., 2012). There is a lot of controversy related to the involvement of biotic agents as well as abiotic factors (Rossetti, 2001; Auler et al., 2011). That implicates in the inexistence of prophylactic measures for the control of the citrus blight, so that the only recommended practice is the removal of the sick plants when they enter into an economically unviable production phase.

Physiological alterations associated with plant stress responses are, at many times, controlled at a transcriptional level, thus, differentially expressed genes in plants affected by the blight possibly possess an associated function to this disease. As such, the identification and the quantification of such genes in affected plants can supply important information on the physiological and molecular processes involved in the defense response of the citrus against the citrus blight.

The suppressive subtractive hybridization technique (SSH) allows the retrieval of small cDNA libraries enriched with differentially expressed transcripts that are present in just one of the compared samples (Diatchenko et al., 1996). This tool has been used in an efficient way to obtain this sort of information in various pathosystems, such as in the identification of genes induced in response to infection of plants by pathogens (Horwitz; Lev, 2010; Hirao; Fukatsu; Watanabe, 2011) and of genes induced in response to abiotic stress (Ouyang et al., 2007; Basyuni et al., 2011). Therefore, the objectives of this study were to identify and quantify genes differentially expressed in plants affected by the citrus blight, through the techniques of suppressive subtractive hybridization and real time PCR, seeking to support the development of new control strategies for this disease, as well as the enhancement of early diagnosis and the obtainment of resistant plants.

MATERIAL AND METHODS

Material collection

Samples of "Cravo" lemon tree (*Citrus limonia* L. Osbeck) roots were collected under "Pera" orange (*Citrus sinensis* L. Osbeck) in a farm located in the city of Bebedouro, SP. The samples were removed from trees with approximately 12 years of age, originating from the same stand and submitted to the same cultivation conditions. Based on typical citrus blight visual symptoms and on the syringe test (Lee et al., 1984), the trees, whose the roots were sampled, were classified in 3 stages: healthy (without apparent symptoms of the disease), beginning of the disease (plants exempt of new shoots, leaves with opaque green coloration, defoliation and dry leaf tips) and advanced stage (plants exempt of new shoots, except inside the primary branches, leaves with opaque green coloration, abundant defoliation and severely dry crown branches). Afterwards, surface roots of each tree were collected for RNA extraction. These samples were stored in a freezer at -80° C until the time of the analyses.

RNA extraction and purification of mRNA

Total RNA of the roots of sick and healthy plants was isolated by the Trizol method (Chomczynsky; Sacchi 1987). Later on, total RNA was treated with *Deoxyribonuclease I Amplification Grade* (Invitrogen™) and the mRNA purified with the *Oligotex mRNA Spin-Column Protocol* (QIAGEN) kit, according to manufacturer specifications.

Suppressive Subtractive Hybridization

The PCR of cDNA subtraction was conducted by using the *PCR-Select™ cDNA Subtraction Kit* (Clontech). The two cDNA subtraction libraries were built starting from 2 μg of mRNA, according to the manufacturer protocol. For the forward subtraction, the mRNA of the sick plant was used as tester, and of the healthy plant as a driver. For the reverse subtraction, the mRNA of the healthy plant was used as tester, and of the sick plant as a driver. Soon afterwards, the cDNA tester of both was digested with *Rsa*I and the insert bonded to the adapters 1 and 2R. The hybridization and amplification processes were conducted twice with the intention of increasing the number of differentially expressed sequences. The subtracted cDNAs obtained from the second amplification were cloned in *pDrive Cloning Vetor* (Qiagen® PCR Cloning Kit) and transformed in E. *coli DH5α™ T1 Phage Resistant* (Promega). The transformants were placed on LB medium containing ampicillin [100 μg/mL], x-gal [40 mg/mL] and IPTG [100 mg/mL] and the confirmation of the bonding of the insert was given via PCR of the colonies transformed using the M13primers.

Sequencing and analysis of SSH cDNAs library

After transformation, 129 bacterial colonies selected for the sequencing were incubated overnight in liquid LB medium according to Sambrook, Fritsch and Maniatis (1989) and the plasmids were extracted by alkaline lysis (Birnboim; Doly, 1979). The cDNA inserts were sequenced (Mega Bace 1000-Amersham Biosciences) by the method of Sanger, Nicklen and Coulson (1977). The obtained sequences were analyzed using the Blast X algorithm ion the National Center for Biotechnology Information (NCBI) databases (http://www.ncbi.nlm.nih.gov/BLAST/) and those of the Centro de Citricultura de Cordeirópolis (http://www.centrodecitricultura.br/).

Analysis of gene expression

The evaluation of the candidate genes expression patterns was conducted by reverse (RT) and quantitative PCR (RT-qPCR) using the SYBR® Green system (Applied Biosystems). The reaction conditions were: 5 min at 50º C, 10 min at 95º C, followed by 40 cycles of 15s at 95º C and 1 min a 60º C, concluding for 15s at 95º C. The expression data was analyzed using the software of the apparatus (version 2.0.1), first being normalized with endogenous control genes, Actin and β-tubulin and later quantified relatively ($RQ = 2^{-\Delta\Delta CT}$) according to Livak and Schmittgen (2001). The efficiency of the reactions was checked by the equation $(1+E)=10^{(-1/slope)}$ (Ramakers et al., 2003). The following primers (which obtained an efficiency higher than 0.86) were used for validation of the expression analysis (forward; reverse): 14-3-3 (5'-TCCCTATTGGTGGGTGAACAA-3',5'-GATGTCGGCTCTTCCTATCATTG-3'); Cytochrome P450 cTBP type (5'-TCC AGCGAAACCACAGCAA-3', 5'-AAGCTCAACAGGGTCTTCTTTCC-3'); Senescence Protein (5'TCCCTATTGGTGGGTGAACAA3', 5'GATGTCGGCTCTTCCTATCATTG3'); β-tubulin (5'GCGTCACATGCTGTCTTCGAT3', 5'GCGGGAAAGGGATAAGGTTAA3'); Actin (5'GCCACACAGTCCCAATCTATGA3', 5'GATCACGACCGGCA AGGT3').

RESULTS AND DISCUSSION

The identification and quantification of the differential gene expression of genes related to the citrus blight can be a key-factor to reveal the causal agent and the action mechanisms of this anomaly that causes great damage to citriculture. In this work, two subtractive libraries were built, one enriched with cDNAs differentially expressed in sick plants (forward) and another enriched with cDNAs differentially expressed in healthy plants (reverse). 38 clones of the forward library and 91 of the reverse library were obtained (Table 1 and 2), that were identified, by database comparison, as sequences associated to stress processes. After identification, three genes were selected (Probable senescence related protein; Monooxigenase of the cytochrome P450 - Type cTBP, related to abiotic stress and a protein of the 14-3-3 family, related to disease resistance) for quantitative analysis of the genic expression in different evolutionary stages of the citrus blight anomaly.

After the database search, sixteen clones of the forward library and eighteen of the reverse presented similarity with a probable protein related to the senescence in snow pea-pods – ssa13 (Pariasca et al., 2001). This gene is downstream regulated, presents low transcription level in the leaves and immature pods and it does not present homology with known database sequences (Pariasca et al., 2001). Senescence has been associated to genes that econde pathogenesis related proteins (Lers, 2007), degrading enzymes (Drake et al., 1996) and stress response proteins (Sharabi-Schwager et al., 2010). As such, based on the results of the relative quantitative expression (Figure1) there is the possibility that this gene, homologous to ssa13, has some function in the defense process of the plant against stress caused by the blight since the sick plants presented a relative expression approximately twofold higher than the healthy plants. However, despite the senescence being characterized by the activation and inactivation of different groups of genes (Gan; Amasino, 1997), the inactivation of genes, in itself, is not enough to cause the senescence, because the senescence process can be blocked by RNA inhibitors, protein synthesis and enucleation (Drake et al., 1996, Gan; Amasino, 1997).

Table 1 – Genes expressed in roots of sick plants and absent in healthy plants, gi: gene index indicating name of EST, the homology of that sequence with the NCBI (National Center for Biotechnology Information - http://www.ncbi.nlm.nih.gov) database, Size: size of the clone with highest number of nucleotides sequenced, E-value: identity with sequences of the database and number of clones. Number of clones identified with the same annotation.

Gi	Homology (NCBI)	Size (pb)	E-value	Nº of ESTs
gi|13359451|dbj|BAB33421.1|	probable senescence associated protein	861	2e-42	16
gi|82734183|emb|CAJ44125.1|	dehydrin type LEA protein	654	9e-07	7
gi|1545805|dbj|BAA10929.1|	cytochrome P450 type cTBP protein	715	2e-43	1
gi110740129|dbj|BAF01964.1|	hypothetical protein	866	3e-15	6
	without significant similarity	643		8

Table 2 – Genes expressed in roots of healthy plants and absent in sick plants, Gi: gene index indicating name of EST, the homology of that sequence with the NCBI (National Center for Biotechnology Information - http://www.ncbi.nlm.nih.gov) database, Size: size of the clone with highest number of nucleotides sequenced, E-value: identity with sequences of the database and number of clones. Number of clones identified with the same annotation.

Gi	Homology (NCBI)	Size (pb)	E-value	Nº of ESTs
gi|13359451|dbj|BAB33421.1|	probable senescence associated protein	861	2e-42	18
gi|82734183|emb|CAJ44125.1|	dehydrin type LEA protein	654	9e-07	13
gi|1545805|dbj|BAA10929.1|	cytochrome P450 type cTBP protein	715	2e-43	11
gi|16225430|gb|AAL15887.1|AF417299.1	possible germin	630	1e-06	6
gi|15232958|ref|NP_186921.1|	Hydrolase	406	4e-08	2
gi|2760345|gb|AAB95250.1|	Ubiquitin	698	1e-59	2
gi|3308980|dbj|BAA31561.1|	metallothionein type protein	711	8e-12	2
gi|8099061|gb|AAD27824.2|	14-3-3 protein	611	8e-27	4
gi|89282373|gb|EAR81082.1|	hypothetical protein	866	1e-24	8
	without significant similarity	862		25

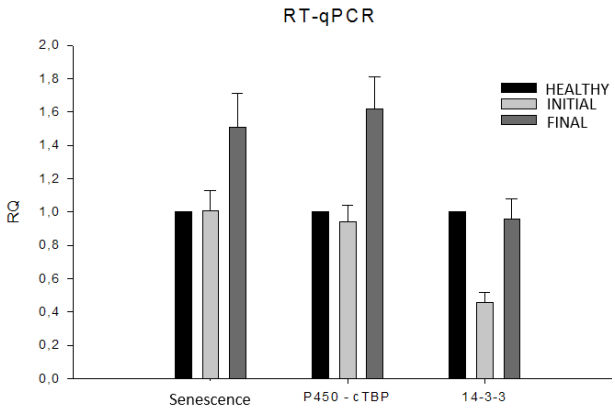

Figure 1 – Relative quantitative expression (RQ) profile by RT-qPCR of the candidate gene. Columns represent the expression of transcripts obtained from Citrus root in different stages of blight. The transcript abundance was normalized by the expression of the Actin and β-tubulin genes. Transcripts of the healthy condition were used as reference sample.

One clone of the forward library and eleven of the reverse, presented similarity with the cytochrome P450 protein of the type cTBP from tobacco, which s related, in higher plants, to the enzymatic activity in the xenobiotic metabolism (Sugiura et al., 1996). This protein presents only 22% of identity with P450 of other plant species (Sugiura et al., 1996), but the conserved region of the heme group that contains 16 amino acid residues, presents five residues (leucine, phenylalanine, serine, cysteine and alanine) identical to those of *Persea americana* (Bozak et al., 1990) and *Zea mays* (Larkin, 1994). As such, it suggests that the P450 protein of type cTBP is not microssomal, but can be present in the mitochondria, in the chloroplast or soluble (Sugiura et al., 1996). In plants, the development and the hormonal levels regulate the expression of many members of the cytochrome P450, furthermore, various xenobiotic inductors can also regulate the genic expression of P450 at the transcriptional level (Mendoza, 2009). In plants, the P450 transcriptional levels can be altered by some factors such as wounds (Whitbred; Schuler, 2000) and chemical agents (Yamada, 2000). The monooxygenase enzymes of the cytochrome P450 are the main enzymes involved in the initial phase of the xenobiotic metabolism (Timbrell, 2000). Liu et al. (2003) reported that the cytochrome P-450 has a high expression during the interaction of mycorrhizal fungi with plant roots. In this experiment, the relative expression of the gene homologous to the cytochrome P-450 of the type cTBP in the citrus blight increased as the disease developed (Figure 1). The gene homologous to the cytochrome P450 presented expression of approximately 1.8 fold higher than in relation to the initial stage (Figure 1),

in the final stage of the disease. Mendoza (2009) also reported that the P-450 family enzymes are related to the biosynthesis of important metabolites in plant response to pathogen and herbivore attack and that the insertion of cytochrome P-450 genes, via transgenic techniques, increases the possibility of obtaining plants more tolerant to stress. As such, the enzyme found in this experiment is possibly related to the citrus blight anomaly, being a good candidate for the study of blight.

Four clones of the reverse library presented similarity with the protein 14-3-3 of poplar (Lapointe et al., 2001). The 14-3-3 proteins are from a family of regulatory molecules, with 9 to 10 conserved domains of the α-helix type (Lapointe et al., 2001) that modulate various cellular processes such as cell cycle, metabolism, stress response and signal transduction (Aitken, 2006; Shin et al., 2011). The association of those proteins with the G-box binding factor suggests that they are directly involved in transcriptional regulation (Kuramae; Fenille; Jr 2001). In sugarcane, these proteins were expressed in all of the cDNA libraries obtained from different tissue (meristem, callus, flower, sprout, leaves, root, stalk, seed) and of seedlings infected with bacteria (Kuramae; Fenille; Jr, 2001). However, in this experiment, healthy and sick plants presented similar levels of expression for this gene (Figure1), so maybe an eventual flaw occurred in the subtraction process leading to the amplification of segments that are not differentially expressed.

CONCLUSIONS

In this study the suppressive subtractive hybridization technique (SSH) was used to obtain subtractive libraries, which resulted in 129 clones of differentially expressed cDNA in response to the citrus blight. The identification and quantification of these genes generated important data that will help in the development of new strategies to control citrus blight, among which are the development of early detection methods for sick plants and the obtaining of more resistant plants. The marker-assisted selection and the obtaining of transgenic plants overexpressing or knocking out some of those genes is a promising alternative that can impel the citrus blight research.

REFERENCES

AITKEN, A. 14-3-3 proteins: a historic overview. **Seminars Cancer Biology**. 16(3):162-172, 2006.

AULER, P. et al. Ocorrência de declínio dos citros na região Noroeste do Paraná. **Revista Brasileira de Fruticultura**. 33(1):286-290, 2011.

BALDASSARI, R. B.; GOES, A. de; TANNURI, F. Declínio dos citros: algo a ver com o sistema de produção de mudas cítricas. **Revista Brasileira de Fruticultura**. 25(2):357-360, 2003.

BARRIOS, S. et al. Obtenção de anticorpos policlonais contra proteínas presentes em plantas afetadas pela anomalia declínio dos citros. **Ciência e Agrotecnologia**. 30(5):1013-1016, 2006.

BASYUNI, M. et al. Isolation of salt stress tolerance genes from roots of Mangrove plant, *Rhizophora stylosa* Griff., using PCR-Based suppression subtractive hybridization. **Plant Molecular Biology Reporter**. 29(3):533-543, 2011.

BIRNBOIM, H.C; DOLY, J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. **Nucleic Acids Research**. 7(6):1513-1523, 1979.

BOZAK, K.R. et al. Sequence analysis of ripening-related cytochrome P-450 cDNAs from avocado fruit. **Proceedings of the National Academy of Sciences**. 87:3904-3908, 1990.

BRLANSKY, R. et al. **Florida Citrus Pest Management Guide: Blight**, 2012. Available in: < http:// edis.ifas. ufl.edu/ pdffiles/ CG/ CG03800.pdf >. Access in: 22 ago. 2012.

CHOMCZYNSKY, P.; SACCHI, N. Single-step method of RNA isolation by acid guanidinium thiocyanate – phenol – chloroform extraction. **Analytical Biochemistry**. 162(1):156-159, 1987.

DIATCHENKO, L. et al. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. **Proceedings of the National Academy of Sciences of the United States of America**. 93: 6025-6030, 1996.

DRAKE R, et al. Isolation and analysis of cDNAs encoding tomato cysteine proteases expressed during leaf senescence. **Plant Molecular Biology**. 30(4):755-767, 1996.

GAN, S.; AMASINO, R. Making sense of senescence -molecular genetic regulation and manipulation of leaf senescence. **Plant Physiology**. 113(2):313-319, 1997.

HIRAO, T.; FUKATSU, E.; WATANABE, A. Characterization of resistance to pine wood nematode infection in *Pinus thunbergii* using suppression subtractive hybridization. **BMC Plant Biology**. 12(1):12-13, 2012.

HORWITZ, B. A.; LEV, S. Identification of differentially expressed fungal genes in plant by suppression subtraction hybridization. **Methods in Molecular Biology**. 638(1):115-23, 2010.

KURAMAE, E. E.; FENILLE, R. C.; ROSA JUNIOR, V. E. Identification of 14-3-3-like protein in sugarcane (*Saccharum officinarum*). **Genetics and Molecular Biology**. 24(1/4):43-48, 2001.

MENDOZA, D. Complejo enzimático citocromo p450 monooxigenasa en plantas. **Agricultura Técnica En México**. 35(2):225-231, 2009.

LAPOINTE, G. et al. 14-3-3 gene family in hibrid poplar and its involvement in tree defence against pathogens. **Journal of Experimental Botany**. 52(359):1331-1338, 2001.

LARKIN, J.C. Isolation of a cytochrome P450 homologue preferentially expressed in developing inflorescences of *Zea mays*. **Plant Molecular Biology**. 25(3):343-353, 1994.

LEE, R.F. et al. Syringe injection of water into the trunk: a rapid diagnostic test for citrus blight. **Plant Disease**. 68(6):511-513, 1984.

LERS, A. Environmental regulation of leaf senescence. In: GAN, S. **Senescence processes in plants**. Oxford. 1:109-144, 2007.

LIVAK, K.J.; SCHMITTGEN, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-DeltaDelta C(T)) method. **Methods**. 25(4):402-408, 2001.

LIU, J. et al. Transcript profiling coupled with spatial expression analyses reveals genes involved in distinct developmental stages of an arbuscular mycorrhizal symbiosis. **Plant Cell**. 15(9):2106-2123, 2003.

OUYANG, B. et al. Identification of early salt stress response genes in tomato root by suppression subtractive hybridization and microarray analysis. **Journal of Experimental Botany**. 58(3):507-520, 2007.

PARIASCA, J.A.T. et al. Cloning of cDNAs encoding senescence-associated genes, ACC synthase and ACC oxidase from stored snow pea pods (*Pisum sativum* L. var *saccharatum*) and their expression during pod storage. **Postharvest Biology and Technology**. 22(3): 239-247, 2001.

RAMAKERS, C. et al. Assumption free analysis of quantitative real-time polymerase chain reaction (PCR) data. **Neuroscience Letters**. 339(1):62-66, 2003.

ROSSETTI, V. **Manual ilustrado de doenças dos citros: doenças de causas desconhecidas.** Piracicaba: Fealq/Fundecitrus, 2001. 270p.

SAMBROOK, J.; FRITSCH, E.F.; MANIATIS, T. **Molecular Cloning: a laboratory manual**. 2nd ed. New York: Cold Spring Harbor Laboratory Press, 1989. 1659p.

SANGER, F.; NICKLEN, S.; COULSON, A. R. DNA sequencing with chainterminating inhibitors. **Proceedings of the National Academy of Sciences**. 74(12):5463-5467, 1977.

SHARABI-SCHWAGER, M. et al. Overexpression of the *CBF2* transcriptional activator in *Arabidopsis* delays leaf senescence and extends plant longevity. **Journal of Experimental Botany**. 61(1):261-273, 2010.

SHIN, R. et al.14-3-3 Proteins fine-tune plant nutrient metabolism. **FEBS Letters**. 585(1):143-147, 2011.

SUGIURA, M. et al. Cloning and expression in *Escherichia coli* and *Saccharomyces cerevisiae* of a novel tobacco cytochrome P-450-1ike cDNA. **Biochimica et Biophysica Acta**. 1308(3):231-240, 1996

TIMBRELL, J. **Principles of biochemical toxicology.** 3. ed. London: Taylor & Francis, 2000. 394p.

YAMADA, T. et al. Molecular cloning of novel cytochrome P450 species induced by chemical treatments in tobacco cells. **Pesticide Biochemistry and Physiology**. 68(1):11-25, 2000.

WHITBRED, J. M.; SCHULER, M. A. Molecular characterization of *CYP73A9* and *CYP82A1* P450 genes involved in plant defense in pea. **Plant Physiology**. 124(1):47-58, 2000.