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Research Article

Genes associated with hypersensitive response (HR) in the citrus EST database (CitEST)

Simone Guidetti-Gonzalez[1], Juliana Freitas-Astúa[2,4], Alexandre Morais do Amaral[3,4], Natália F. Martins[3], Angela Mehta[3], Marilia Santos Silva[5] and Helaine Carrer[1]

[1]*Departamento de Ciências Biológicas, Escola Superior Luis de Queiróz, Universidade de São Paulo, Piracicaba, SP, Brazil.*
[2]*Embrapa Mandioca e Fruticultura Tropical, Cruz das Almas, BA, Brazil.*
[3]*Embrapa Recursos Genéticos e Biotecnologia, Brasília, DF, Brazil.*
[4]*Centro APTA Citros Sylvio Moreira, Instituto Agronômico de Campinas, Cordeirópolis, SP, Brazil.*
[5]*Embrapa Cerrados, Planaltina, DF, Brazil.*

Abstract

Plants are continuously exposed to pathogen attack, but successful infection is rare because they protect themselves against pathogens using a wide range of response mechanisms. One of them is the hypersensitive response (HR), which is a form of cell death often associated with plant resistance to pathogen infection to prevent the spreadsebpg@cnpq.brsebpg@cnpq.br of the potential pathogen from infected to uninfected tissues. Cell death is activated by recognition of pathogen-derived molecules by the resistance (*R*) gene products, and is associated with the massive accumulation of reactive oxygen species (ROS), salicylic acid (SA), and other pro-death signals such as nitric oxide (NO). The analysis of the citrus EST (CitEST) database revealed the presence of putative genes likely to be involved in HR through their products, like metacaspases, lipoxygenases, phospholipases, pathogenesis-related proteins, glutathione transferases/peroxidases, enzymes involved in the phenylpropanoid pathway and in the formation and detoxification of ROS, as well as those involved in the formation and regulation of ion channels, SA and NO. By analysis of the EST database of Citrus, it was possible to identify several putative genes that code for key enzymes involved in HR triggering and also in plant defense against biotic and abiotic stress.

Key words: otic and abiotic stress response, plant-pathogen incompatible interaction, programmed cell death, plant disease resistance.

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Introduction

Plant-pathogen interactions involve complex, specific, and dynamic mechanisms. When a plant is resistant to a pathogen (incompatible interaction), its recognition often results in a hypersensitive reaction (HR), a rapid cell death of the plant tissues at the infection site that contributes to the limitation of growth and spread of the pathogen (Gabriel and Rolfe, 1990; Parker and Coleman, 1997; Lee and Hwang, 2005). It is generally believed that recognition of incompatible pathogens, via specific resistance genes encoded by the plant genome, leads to the activation of a number of different signaling mechanisms that initiate at the plasma membrane (Pontier *et al.*, 2002).

Send correspondence to Helaine Carrer. Departamento de Ciências Biológicas, Escola Superior Luis de Queiróz, Universidade de São Paulo, Av. Pádua Dias 11, 13418-900 Piracicaba, SP, Brazil. E-mail: hecarrer@esalq.usp.br.

The initial events following the recognition of the pathogen elicitor by the plant are calcium influx, alkalinization of the extracellular space, protein kinase activation, production of reactive oxygen species (ROS) and nitric oxide (NO), and transcriptional reprogramming (Dangl and Jones, 2001). The H_2O_2 produced by the oxidative burst in the dark does not play an important role in the execution of cell death, but an early and massive production of fatty acid hydroperoxides by lipoxygenases has a prominent role in cell death (Montillet *et al.*, 2005). NO and ROS could also contribute to rapid transcriptional activation of a battery of "defense genes" in and surrounding the infected cell. Some of these genes encode peroxidases, glutathione *S*-transferases, proteinase inhibitors, and various biosynthetic enzymes, such as phenylalanine ammonia lyase (PAL), which is the first enzyme in the phenylpropanoid pathway and is involved in the synthesis of low molecular weight, antimicrobial compounds known as phytoalexins

(Klessig *et al.*, 2000). Other defense mechanisms that are activated include biosynthesis of salicylic acid, induction of ethylene biosynthesis, cell-wall strengthening, and lignification (Scheel, 1998; Lam *et al.*, 2001). The oxidative burst leads to a HR and then to the establishment of systemic acquired resistance (SAR) (Rentel *et al.*, 2004).

HR has been a source of interest and controversy since its recognition in 1915; however, in recent years, the combination of molecular genetics, computer-enhanced microscopy, new cytochemical techniques and a better understanding of plant signal transduction and cell biology have helped to unravel this apparently universal plant response (Heath, 2000).

A functional genomics project has been initiated to study the molecular characterization of the main biological and agronomical traits of citrus (Forment *et al.*, 2005) and the present work also tries to help to uncover the genetic machinery of *Citrus* species. The objective of this work was to identify expressed sequence tags (ESTs) involved in the codification of key enzymes related to the HR in citrus plants. By searching the citrus EST database (CitEST), we describe putative pathways that may lead to HR in citrus plants.

Materials and Methods

In the Citrus EST Sequencing Project (Millennium Project-CNPq), various libraries of citrus cDNA were constructed with tissues from *Citrus sinensis, C. reticulata, C. limonia, C. aurantifolia, C. aurantium, C. limettiodes, C. latifolia, C. sunki* and *Poncirus trifoliata*, in different combinations of limiting factors (biotic and abiotic), physiological conditions (adult x juvenile) or tissue (leaf, bark, fruit, root, flower, and seed). The libraries description can be found in Targon *et al.* (this issue). The CitEST database contains over 181,000 sequenced ESTs (see CitEST: http://biotecnologia.centrodecitricultura.br), which were used in this work.

The identification of the genes related to HR was performed by using keywords (metacaspases, calcium and potassium channels, NAD(P)H oxidase, peroxidases, superoxide dismutase, nitrate reductase, chorismate mutase, phenylalanine ammonia-lyase, cinnamic acid 4-hydroxylase, chalcone synthase, isochorismate synthase, lipoxygenase, phospholipase, glutathione transferase, glutathione peroxidase, chitinase, osmotin, thaumatin, permeatin) and by BLASTX searches with related sequences present in other species, like *Arabidopsis thaliana*, *Lycopersicon esculentum* (tomato), *Oryza sativa* (rice), *Nicotiana tabacum* (tobacco) and others in the CitEST database. Reads with E value < 10^{-10} were individually inspected, excluding sequences unrelated to the target enzyme, whereas the remaining sequences were clustered using the CAP3 (Huang and Madan, 1999). In order to analyze phylogeny among CitEST contigs and coding region of the proteins identified in the NCBI database of known HR genes, cladograms were made using ClustalW program (http://www.ebi.ac.uk/clustalw/).

Results and Discussion

The clusterization of the reads identified using keywords and BLASTX with known HR genes from other plant species in the CitEST database resulted in 227 contigs and 174 singletons. Table 1 shows 18 proteins involved in the HR process identified in the CitEST, including the total number of contigs and singletons obtained. The presence of these proteins permitted inference of a putative pathway that leads to HR in citric plants.

The NCBI database (http://www.ncbi.nlm.nih.gov/) has 184,881 and 28,822 nucleotides sequences of *Citrus* and *Poncirus* species, respectively. Searches in this database revealed the presence of two sequences related to glutathione peroxidase, one peroxidase, two superoxide dismutase, five phenylalanine ammonia-lyase, three cinnamic acid 4-hydroxylase, three chalcone synthase, three lipoxygenase, four chitinase and one osmotin in *Citrus* sequences, while for the *Poncirus* species there are just one superoxide dismutase, two nitrate reductase and one glutathione transferase. Some HR genes were not identified in this database, such as metacaspase, calcium and potassium channel related proteins, nitrate reductase, isochorismate synthase, chorismate mutase, phospholipase and glutathione transferase, however they were identified in the CitEST database. According to these data, this work would be con-

Table 1 - Classes of HR proteins identified in CitEST database.

HR Proteins	Total number of	
	Contigs	Singletons
Chalcone synthase	4	12
Chitinase	29	24
Chorismate mutase	5	4
Cinnamic acid 4-hydroxylase	3	2
Glutathione peroxidase	10	2
Glutathione transferase	36	4
Isochorismate synthase	2	1
Lipoxygenase	16	18
Metacaspase	3	4
Nitrate reductase	3	1
NADPH oxidase	3	3
PR-5-like proteins	10	4
Peroxidase	52	42
Phenylalanine ammonia-lyase	4	7
Phospholipase	9	12
Potassium channel	17	16
Superoxide dismutase	19	16
Two-pore calcium channel	2	2

sidered the first attempt to catalog the majority of genes related to HR in *Citrus* and *Poncirus* species.

The identification of resistance (*R*) genes that permits plants to perceive the pathogen invasion and trigger the ion flux, which culminates in HR, complements this work, and is reported in Guidetti-Gonzalez and Carrer (this issue). Several distinct MAPK (mitogen-activated protein kinases) cascades have been implicated in the regulation of plant disease resistance through signal transduction between perception of the elicitors and the activation of disease resistance genes. These MAPKs are reported in Mehta *et al.* (this issue). Besides, several pathogenesis related (PR) proteins are known to be expressed in the context of HR and usually act as antifungal factors at the end of the HR signaling cascade. Herein, the expression of two types of PR proteins typically presenting antifungal activity (chitinases and osmotins/thamatins/permeatins) were analyzed within the CiEST database. Campos *et al.* (this issue) are presenting a complete analysis of citrus PR proteins under biotic and abiotic stress.

The proteins involved in HR, found in the present work, are detailed below:

Metacaspases

Apoptosis in animals is characterized and commonly defined by the activation of a family of cysteine-dependent aspartate-specific proteases, or caspases (Pennell and Lamb, 1997). Caspases can proteolytically activate downstream caspases or cut various cellular substrates, leading to cell death (Utz and Anderson, 2000). Caspase homologues in plants have been reported and called metacaspase (Hoeberichts *et al.*, 2003; Vercammen *et al.*, 2004). In tomato, a metacaspase *LeMCA1*, is induced during infection of leaves with *Botrytis cinerea* (Hoeberichts *et al.*, 2003). Searching within the CitEST, we found 18 reads that match with metacaspases of *A. thaliana*, *L. esculentum* and *O. sativa,* which formed three contigs and four singletons (Table S1). One of these singletons, with similarity of 3e-84 to *L. esculentum,* came from *P. trifoliata Citrus tristeza virus*-infected leaf library, which suggests involvement in plant disease resistance, as observed in other species (De Jong *et al.*, 2000; Hoeberichts *et al.*, 2003).

Calcium and Potassium channels

Some of the earliest signaling events detected during the HR include ion fluxes, influx of calcium and H^+, and efflux of K^+ (Atkinson *et al.*, 1990).

Calcium, as the divalent cation (Ca^{2+}), is an essential plant nutrient. It is required for structural roles in the cell wall and membranes, for inorganic and organic anion uptake in the vacuole, and as an intracellular messenger in the cytosol. Polarization or depolarization of the membrane voltage opens the gate subunits and allows calcium flow through the cell membrane. When calcium ions enter plant cells through Ca^{2+}-permeable ion channels, tonoplast and/or endoplasmic reticulum, cellular responses to a diverse range of developmental cues and environmental challenges are initiated (White, 2000; Sanders *et al.*, 2002).

The search for calcium channel sequences within the CitEST database revealed the presence of 15 reads (Table S1). The clustering identified as potential calcium channels, two contigs and two singletons, contig 1 (C1) was mainly composed of *C. sinensis* sequences with only one sequence from *P. trifoliata* and one occurrence of *C. reticulata*. Contig 2 (C2) was mostly composed of *P. trifoliata* sequences (two reads) and one read from *C. reticulata*. Singletons were found in *C. limonia* and *P. trifoliata*.

Contig 1 showed 75% identity of deduced protein sequence with the Two-pore channel 1 (TPC1) from *Arabidopsis thaliana* (Furuichi *et al.*, 2001). Contig 2 showed 61% amino acid identity with the Two-pore calcium channel TPCB1 from *N. tabacum*. One singleton is 74% identical to the *N. tabacum* TPC and the other is 50% identical to the tobacco TPC1B.

The wheat channel homologue to TPC1 from *A. thaliana* was cloned and expressed under abscisic acid (ABA) and various stress treatments indicating that these channels might be the first gate of control for several cell phenomena related to Ca^{2+} influx as cell signaling, homeostasis and stress response (Wang *et al.*, 2005).

Potassium is the predominant inorganic ion of plant cells, where it plays a major role in contributing to cellular hydrostatic (turgor) pressure, growth, and responses to the environment (Michard *et al.*, 2005).

The *HLM1* gene encodes a member of the CNGC (cyclic nucleotide - gated channel - CNGC4) ion channel family (Maser *et al.*, 2001). The study of this gene product showed that CNGC4 is permeable to both K^+ and Na^+, is activated by cGMP and cAMP, and its expression is induced in response to pathogen infection and some pathogen-related signals. So HLM1 may constitute a common downstream component of the signaling pathways leading to HR (Balague *et al.*, 2003). In our analyses, we found only one singleton similar to HLM1 (6e-96), the other 15 singletons and 17 contigs showed similarity to other members of the CNGC family (Table S1), possibly with potassium channel activity. Our comparative analysis of the potassium channel revealed its presence in the *C. reticulata*, *C. sinensis*, *C. latifolia*, *C. aurantifolia* and *P. trifoliata* libraries. These results were expected because potassium is involved in wide environmental responses and is possibly involved in the citrus HR as well.

Reactive oxygen species

The generation of reactive oxygen species (ROS), such as singlet oxygen, superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals is one of the earliest responses during HR just after ion fluxes (Atkinson *et al.*, 1990). These ROS not only have direct antimicrobial activ-

ity (Lamb and Dixon, 1997), but also act as signaling molecules leading to up- or down-regulation of numerous genes involved in plant defense responses such as the induction of defense-related genes and initiation of programmed cell death (PCD) at the site of the attempted infection (Neill *et al.*, 2002; Apel and Hirt, 2004). This localized PCD generates a physical barrier restraining nutrient availability because of the rapid dehydration caused by tissue death (Parker and Coleman, 1997).

Plants have a number of ways of generating extracellular ROS (Bolwell and Wojtaszek, 1997). During the incompatible pathogen interaction, superoxides are produced enzymatically outside the cell and are rapidly converted to H_2O_2, which is able to cross the plasma membrane (Melillo *et al.*, 2006). Cell wall-bound peroxidase (POXs) and a plasma membrane-bound NADPH oxidase are considered the major sources for ROS production as defense mechanisms during biotic stresses (Heath, 2000; Kawano, 2003; Melillo *et al.*, 2006), even though there is evidence supporting a differential ROS generation for different stimuli as well (Bolwell, 1999).

The balance of production and detoxification of ROS is essential for aerobic life (Apel and Hirt, 2004) and the key enzymes or enzyme families involved in the production, as well as scavenging of ROS and induction of HR in plants are nicotinamide-adenine dinucleotide phosphate [NAD(P)H] oxidase, peroxidases and superoxide dismutases. Superoxide radicals generated by the one-electron reduction of molecular oxygen are rapidly converted within the chloroplast to hydrogen peroxide by CuZn-superoxide dismutase (Asada, 1999). The superoxide dismutases (SODs) are extremely effective antioxidant plant enzymes (Skinner and Baek, 2006), since they dismutate two superoxide anions into H_2O_2 and O_2 (Fridovich, 1991).

Three contigs and three singletons harboring NAD(P)H oxidase gene sequences were obtained from the CitEST database. The three contigs comprised 16, 4 and 5 reads, respectively. They were assembled with ESTs from both *C. sinensis* and *P. trifoliata* libraries. However contig 2 (C2) and contig 3 (C3) presented only reads from the leaf libraries, contig 1 (C1), with the largest number of reads, comprised sequences from fruit tissue as well. The three contigs contained partial gene sequences and exhibited similarity with NADPH oxidase protein from either *Nicotiana* sp. or *A. thaliana* as best BLASTX hits (Table S1).

Plants have multiple genes encoding peroxidases (Tognolli *et al.*, 2002; Apel and Hirt, 2004)**.** Therefore, as expected, a large number of peroxidase transcripts were identified, 568 reads total, which clustered in 52 contigs and 42 singletons (Table S1).

Several interesting inferences could be drawn from these sequences. Considering that glutathione S-transferase/peroxidase comprises a large number of reads, which are from all of the seven *Citrus* and *Poncirus* species and also the six tissues available at the CitEST, we can infer that the expression of these transcripts are constitutive not only in the genus *Citrus* but, likely, in other related rutaceae.

However, in a few cases, the specificity of the contigs is evident, with some particular conditions leading to the clustering of similar sequences in well-defined contigs. One of the contigs (C63) was composed of three *C. limonia* sequences, all of them were from the library constructed from roots undergoing water stress, suggesting that this transcript may code for a peroxidase involved in drought response. Interestingly, three contigs (C22, C64 and C65) were composed by reads from a library constructed from *C. sinensis* symptomatic for citrus variegated chlorosis (CVC), caused by *Xylella fastidiosa*, one of the most devastating diseases of the crop, indicating that peroxidases may be involved in the response of citrus to this particular biotic stress.

The multicopy pattern of superoxide dismutase (*SOD*) gene observed in various hosts was also found within the CitEST database, which yielded 19 contigs and 16 singletons with similarity to translated SOD sequences (Table S1).

As in the peroxidases, at least one contig harbored 43 sequences from most of the species and tissues from where the libraries were constructed. Interestingly, no *SOD* gene was found to be expressed in the flower tissue, but that might be due to the relatively low number of reads of this library (4,330) compared to the others (Targon *et al.*, this issue). On the other hand, some contigs comprised reads from one or few species. While three contigs (C3, C18 and C19) were formed of only *Poncirus* sp. reads, the other 11 were formed of only *Citrus* sp. sequences. Within these latter, some harbored sequences from a particular species (two contigs from *C. reticulata* alone, five from *C. sinensis*, and one from *C. latifolia*), suggest high specificity of these transcripts.

Likewise the peroxidases, but on a lower level, it was possible to determine some tissue-specificity of the *SOD* transcripts. At least six contigs (C3, C4, C12, C13, C18, and C19) harbored sequences only from the leaf libraries, while the other three (contigs C7, C9, and C16) were formed solely by reads from the fruit libraries. Furthermore, few contigs comprised reads from a particular condition. One of them (contig C12), of great interest, was composed of 39 reads from both the *C. sinensis* and *C. reticulata* leaf libraries infected with *X. fastidiosa*. This transcript was similar to a Cu/Zn superoxide dismutase from *C. sinensis* (CAA03881.1) and, since the two species are susceptible and resistant to CVC, respectively, it is likely that such transcript is not involved in disease resistance, but in the response to this specific biotic stress.

Nitric oxide

Nitric oxide (NO) has well-known biological functions in mammals, but it was only recently recognized as a

signal compound in plants (Zhao *et al.*, 2005). In fact, it is now known that NO plays an important role in diverse physiological processes in plants (Grun *et al.*, 2006). The Avr factors from pathogens stimulate NO production, which promotes disease resistance in plants, collaborating with ROS in the oxidative burst (Delledonne *et al.*, 1998). The relative level of NO and H_2O_2 appears to be very important to HR-associated cell death in soybean cells (Delledonne *et al.*, 2001) and it was observed that only the simultaneous increase of NO and H_2O_2 in tobacco cells induced typical programmed cell death (de Pinto *et al.*, 2002).

Two potential enzymatic sources of NO in plants are NO synthase (NOS) and nitrate reductase (NR). NOS is a family of well characterized enzymes in mammalian cells (Desikan *et al.*, 2002) that was found in several plants (Neill *et al.*, 2002), and serves as a signal for plant growth, development, and defense (Delledonne *et al.*, 1998; Klessig *et al.*, 2000; Neill *et al.*, 2002). NR is a central enzyme of nitrogen assimilation in plants (Lea, 1999; Kaiser *et al.*, 2002), and is induced in potato tubers treated with either *Phytophthora infestans* or an elicitor derived from this oomycete pathogen (Yamamoto *et al.*, 2003).

Despite the large number of citrus ESTs sequenced in the CitEST Project, we did not find reads similar to NOS, which suggests that the NO synthesis in these plants can be preferentially performed by NR. Within the CitEST database, three contigs and one singleton similar to plant nitrate reductase were assembled (Table S1). One of these contigs (C10), similar to *Petunia x hybrida* and also *Tilia platyphyllos* (e-values = 0.0) nitrate reductase, seems to be expressed especially within non-infected leaf tissue from *C. reticulata* and *P. trifoliata*, while the unique read from bark *P. trifoliata* is from *Phytophthora*-infected library. These results suggest that this contig has species- and tissue-specific expression, showing that the bark tissue of *P. trifoliata* may be involved in the production of NO for plant defense, as with potato tubers infected with *P. infestans* (Yamamoto *et al.*, 2003).

Another contig (C11), similar to NR from *Tilia platyphyllos* (2e-152) presents reads solely from *C. reticulata* and a tendency of expression in *Xylella fastidiosa*-infected leaves (Figure 1a). This specific expression pattern suggests that this protein is important to plant defense specifically in *C. reticulata*. The third contig (C7) did not exhibit a species-specific expression, but comprised mainly sequences from the *C. reticulata* and *C. sinensis* leaf libraries infected with *X. fastidiosa,* indicating that this transcript may be involved in the response to this biotic stress. Figure 2 shows the estimate of a phylogeny among contigs C7, C10 and C11, with nitrate reductase from *Petunia x hybrida* (gi|294113), *Tilia platyphyllos* (gi|24474445) and *Ricinus communis* (gi|11119240). As expected, it was observed that Contigs C10 and C11 are more closely related to each other and are related with *Tilia platyphyllos* and *Petunia x hybrida*, and C7 is more closely related to *Ricinus communis*.

Phenylpropanoid pathway

In plants, the phenylpropanoid pathway is responsible for the synthesis of a wide variety of secondary metabolic compounds, including lignins, salicylates, coumarins, hydroxycinnamic amides, pigments, and flavonoids (Hwang *et al.*, 2003).

In the plant phenylpropanoid pathway, chorismate mutase catalyses the reaction that forms phenylalanine, and the enzyme phenylalanine ammonia-lyase (PAL) deaminates phenylalanine to yield cinnamic acid (Gozzo, 2003; Kaneko *et al.*, 2003). Several other enzymes are present in the phenylpropanoid pathway, with cinnamate 4-hydroxylase (C4H)-the first cytochrome P450 upstream in this pathway - leading to the synthesis of a variety of compounds including lignin monomers, flavonoids, and furocoumarins (Morant *et al.*, 2003; Gravot *et al.*, 2004). Chalcone synthase (CHS) is an enzyme that catalyses the reaction that leads to the production of flavonoids and phytoalexins (Yang *et al.*, 2002). The final substance produced by this pathway (4,2', 4', 6'-tetrahydroxychalcone) serves as the backbone for the production of a wide array of flavonoid compounds that function in such diverse roles as flower pigmentation, UV protection, signaling, male fertility, and defense against microbial pathogens (Winkel-Shirley, 2001).

Within the CitEST database, we found five contigs and four singletons similar to chorismate mutase (Table S1), some of them showed specific patterns of expression. The contig 4 (C4) that is similar to *A. thaliana* chorismate mutase (accession number AAC73018) showed specific expression in the *C. sinensis* fruit, whereas the contig 6 (C6) (1e-67) (similar to accession number AAM61395) showed expression in *C. reticulata* fruits and in *C. sinensis X. fastidiosa*-infected leaves (Figure 1b). The other three contigs did not show an expression pattern, presenting reads from different libraries. Figure 3 shows a phylogeny analysis among contigs C4, C5, C6 and C7, and other chorismate mutase sequences from NCBI, showing coherence between contigs and the best hit in BLAST search (Table S1).

Four contigs and seven singletons were found in the search for phenylalanine ammonia-lyase (PAL) within the CitEST database (Table S1). The contig 10 presents high similarity (2e-107) with PAL from *Nerium oleander* and was expressed solely in *P. trifoliata Phytophthora*-infected bark (two reads), suggesting its importance in specific defense of this species. Another contig (C8), comprising sequences with similarity to that from *C. limon*, comprised 20 reads from different libraries. In *C. sinensis,* as in *C. reticulata*, reads showed a specific pattern of expression within *X. fastidiosa*-infected leaf libraries. However, PAL expression was also found in *C. reticulata* within the

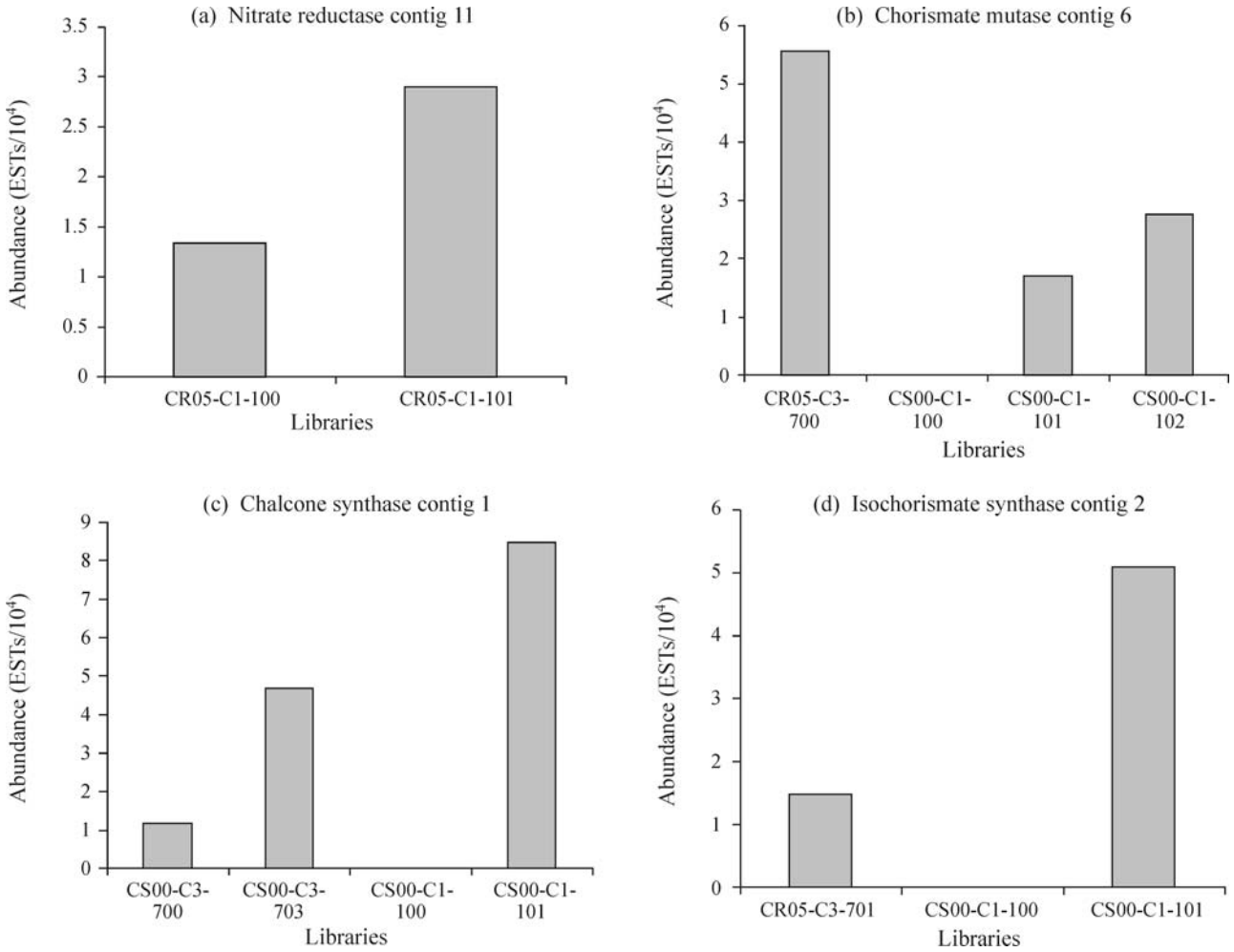

Figure 1 - Transformed data representing the relative abundance of EST by library, expressed in 10^4 reads: (a) Nitrate reductase contig 11, (b) chorismate mutase contig 6, (c) chalcone synthase contig 1, (d) isochorismate synthase contig 2. Code of the libraries: CS: *Citrus sinensis*; CR: *Citrus reticulata*; C1: leaf cDNA; C3: fruit cDNA; 100: non-infected material; 101: infected with *Xylella fastidiosa*; 102: 30 days after *X. fastidiosa* infection; 700: stadium 1 of the fruit; 701: stadium 2 of the fruit; 703 stadium 4 of the fruit.

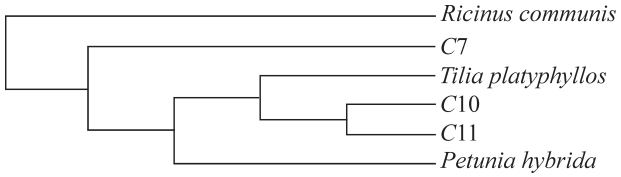

Figure 2 - Cladogram showing phylogeny relation among contigs 7, 10 and 11 with nitrate reductase from *Petunia hybrida* (gi|294113), *Tilia platyphyllos* (gi|24474445) and *Ricinus communis* (gi|11119240).

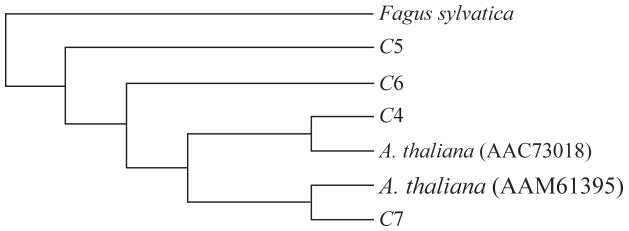

Figure 3 - Cladogram showing phylogeny relation among contigs 4, 5, 6 and 7 with chorismate mutase from *Fagus sylvatica* (ABA54871), *Arabidopsis thaliana* (AAC73018) and *Arabidopsis thaliana* (AAM61395).

healthy fruit library. The contigs C3 (65 reads) and C9 (four reads) were similar to PAL from *Citrus clementina* x *Citrus reticulata* and *Jatropha curcas*, respectively, although they were highly similar to PAL (e-values = 0.0), they did not show any specific expression pattern, since they were formed by reads from a variety of libraries.

In a study with Valencia sweet orange, two cinnamic acid 4-hydroxylase (C4H) cDNAs were cloned and showed differential expression regulation (Betz *et al.*, 2001). One of the cDNAs, C4H2, appeared to be expressed constitutively and seemed to play the role of a `housekeeping' gene in the phenylpropanoid pathway. Conversely, expression studies showed C4H1 to be strongly wound-inducible, but even in wounded tissue it reached much lower levels of mRNA than C4H2 (Betz *et al.*, 2001).

Within the CitEST, we found three contigs and two singletons related to C4H2 (Table S1). One of these contigs (C3) was assembled from 46 reads and showed 100% of identity with C4H2, for which the majority reads originated from *C. sinensis* and *C. reticulata* in different stages of fruit development. Contig 1 (C1) showed 91% of identity with C4H2 and was formed by 11 reads, mostly from *C. sinensis* (10 reads) in the early stages of fruit development and one read from the *P. trifoliata Citrus tristeza virus*-infected library. These results suggest a possible pattern of expression of this protein in the fruits of *C. sinensis* and *C. reticulata.* Moreover, a possible involvement in plant defense against pathogen in *P. trifoliata* cannot be excluded.

In contig 2 (C2), we observed 81% of amino acid identity with *Medicago sativa* C4H protein (Table S1), in agreement with its pattern of expression during fruit ripening (Fahrendorf and Dixon, 1993), since the two reads of this contig were originated from the *C. sinensis* fruit libraries. Figure 4 shows the phylogeny pattern among contigs C1, C2 and C3, and cinnamic acid 4-hydroxylase from *Citrus sinensis* (AAF66066) and *Medicago sativa* (gi|166372); coherence can be observed with the best hit in BLAST search and the phylogenetic relationship.

Two cDNA clones encoding chalcone synthase (CHS) were isolated (CitCHS1 and CitCHS2) from citrus (Moriguchi *et al.*, 1999). The accumulation of CitCHS2 mRNA was notably induced by embryogenesis but CitCHS1 mRNA was not. There was no detectable accumulation of flavonoid in the undifferentiated calli, but flavonoid accumulated after the morphological changes to embryoids. These results indicate that two CHS genes differentially expressed during citrus somatic embryogenesis and CitCHS2 may regulate the accumulation of flavonoids in citrus cell cultures (Moriguchi *et al.*, 1999). Within CitEST, we found four contigs and 12 singletons related to chalcone synthase; among them, three contigs and eight singletons related to CitCHS2. Contig 3 (C3) is formed by 75 reads that mostly came from *C. sinensis* fruits in the early stages of development and is 100% identical to the most homologous BLAST hit (*C. sinensis*). Contig 4 (C4) was composed of 54 reads mostly from *C. reticulata* in the early stages of the fruit development and shares 92% identity with *C. sinensis* CHS. Finally, another contig (C1) was formed by nine *C. sinensis* reads and presented a tendency of expression in early stages of *X. fastidiosa*-infected leaves (Figure 1c), and was 79% identical to *C. sinensis* CHS from NCBI. The singletons related to CHS came especially from *C. sinensis* and *C. reticulata* fruit libraries. Figure 5 shows the expected phylogeny relationship between these contigs and chalcone synthase from *Citrus sinensis* (BAA81664) and *Betula pendula* (CAA71904).

These results indicate a possible presence of at least two CHS genes in *C. sinensis* that are expressed during fruit development as shown in other species (Aharoni and O'Connell, 2002; Kumar and Ellis, 2003). In addition, other CHSs may be involved in citrus response to pathogens. For more information of flavonoid biosynthesis, see Lucheta *et al.* (this issue).

Salicylic acid

The involvement of salicylic acid (SA) as a signal molecule for plant defense has been extensively studied (Shah and Klessig, 1999; Shah, 2003; Stout *et al.*, 2006). SA quickly accumulates at the site of infection during pathogen attack and plant HR, and it spreads to other parts of the plant to induce a wide range of defense responses (Zhao *et al.*, 2005). Besides inducing PR protein genes, SA is assumed to act before the divergence of the two metabolic branches leading to the phenylpropanoid pathway and the activation of the oxidative burst, respectively. In this way, SA amplifies its own synthesis, which, in addition, is stimulated by H_2O_2 (Gozzo, 2003; Zhao *et al.*, 2005).

Previous work has suggested that plants synthesize SA from phenylalanine utilizing chorismate mutase (Gozzo, 2003). However, evidence showed that, in the chloroplast of *Arabidopsis*, SA is synthesized from isochorismate catalysed by isochorismate synthase. This route, typical of bacteria, seems to be required even in other plants to produce SA for defense against pathogens (Métraux, 2002; Gozzo, 2003).

Although evidence from tobacco and potato would indicate that salicylic acid in plants is derived from phenylalanine via benzoic acid, catalysed by benzoic acid 2-hydroxylase (Yalpani *et al.*, 1993; Coquoz *et al.*, 1998) in citrus SA, formation seems to occur via the isochorismate route since the benzoic acid 2-hydroxylase was not found within the CitEST database.

Analysis within CitEST resulted in two contigs and one singleton similar to isochorismate synthases from *M.*

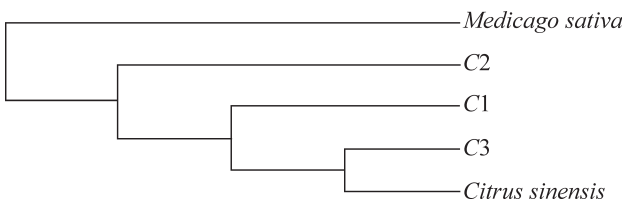

Figure 4 - Cladogram showing phylogeny relation among contigs 1, 2 and 3 with cinnamic acid 4-hydroxylase from *Citrus sinensis* (AAF66066) and *Medicago sativa* (gi|166372).

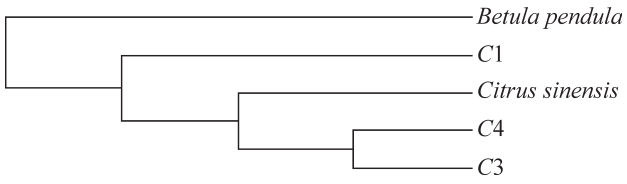

Figure 5 - Cladogram showing phylogeny relation among contigs 1, 3 and 4 with chalcone synthase from *Citrus sinensis* (BAA81664) and *Betula pendula* (CAA71904).

truncatula, *Capsicum annuum* and *Catharanthus roseus* (Table S1). Only one contig (C2), similar to *M. truncatula* isochorismate synthase, showed a specific pattern of expression, presenting a tendency to be expressed in *C. sinensis* leaves, in the early stage of *X. fastidiosa* infection (Figure 1d). This result may suggest that this contig was formed by a transcript that codes for a protein involved in the production of SA upon plant infection by a pathogen.

Lipoxygenases

Lipoxygenases (LOXs) constitute a family of enzymes that deoxygenate polyunsaturated fatty acids containing *cis*, *cis*-1,4-pentadiene structure. The hydroperoxides produced by the LOX reaction are converted into a series of compounds involved in several physiological processes including growth, fruit ripening and response to biotic and abiotic stresses (Hildebrand *et al.*, 1989; Gardner, 1991; Siedow, 1991; Brash, 1999). The most common substrates for plant LOXs are linoleic and linolenic acids, which are important constituents of plant membrane phospholipids. Plant LOXs are classified according to their positional specificity of linoleic or linolenic acid oxygenation, which occurs either at the C-9 or at the C-13 of the hydrocarbon backbone of the fatty acid. Therefore, these LOXs are often referred to as 9-LOXs or 13-LOXs.

Regarding sequence similarity, plant LOXs can be classified into two gene subfamilies. Enzymes with no transit peptide share a similarity higher than 75% with one another and are designated *type 1*-LOXs. The other subfamily, called *type 2*-LOX, contains a putative chloroplast transit peptide sequence and shares a sequence similarity of approximately 35% with each other (Feussner and Wasternack, 2002). In *C. jambhiri*, a LOX containing typical features of a transit peptide for chloroplastic targeting has been identified and expressed upon wounding and inoculation with nonpathogenic isolates of *Alternaria alternata* (Gomi *et al.*, 2002b).

The analysis of the CitEST database revealed 323 sequences related to lipoxygenases, which formed 16 contigs and 18 singletons. The best hit in a BLASTX search as well as the homologous organism are shown in Table S1. The data available from the CitEST database does not allow us to classify the LOXs found and further studies are necessary to characterize the different citrus LOXs. Moreover, most contigs and singletons were incomplete and therefore a classification of the LOXs encountered, based solely on partial sequence comparison, may be premature. The three largest contigs obtained (C3, C7 and C11) revealed deduced amino acid sequences of 921, 945 and 900 amino acids, respectively. The comparison of these sequences with those of the 8 other LOXs showed high similarity to the citrus LOXs (Table S1) revealed that the sequence of contig 7 presented an exclusive amino acid insertion sequence at positions 574 to 606. Contig 7 was formed by reads from healthy and *X. fastidiosa*-infected leaves of *C. reticulata* as well as *X. fastidiosa*-infected leaves of *C. sinensis* and showed 73% identity with *lox1* of *Sesbania rostrata*. Contig 3 showed 71% identity with a *lox2* of *Zantedeschia aethiopica* and contig 11 showed 78% identity with a *lox* of *C. jambhiri*. Contigs 3 and 11 represent complete sequences of the *lox* gene.

Some 9-LOXs have been associated to plant resistance and lipid peroxidation, which is involved in membrane damage and HR (Rance *et al.*, 1998, Kolomiets *et al.*, 2000; Jalloul *et al.*, 2002). The biosynthesis of oxygenated unsaturated fatty acids via LOX pathway may be important for plant defense against pathogen attack (Peng *et al.*, 1994). Most of the reads showing similarity to LOXs were obtained from pathogen-infected or fruit libraries. LOXs were highly expressed in the early stages of *X. fastidiosa*-infected libraries of sweet orange (*C. sinensis*) and mandarin (*C. reticulata*) (up to 30 days after inoculation-DAI). Some ESTs associated with lipoxygenase were also observed in *C. reticulata* up to 60 DAI. Several studies have reported that LOX activity in plants is higher and more rapid upon infection of avirulent pathogens as compared to virulent pathogens (Ocampo *et al.*, 1986; Yamamoto and Tani, 1986; Siedow, 1991; Melan *et al.*, 1993). *C. reticulata* is resistant to *X. fastidiosa* and, as expected, a higher expression of LOX was observed in *X. fastidiosa*-infected mandarin plants. LOX was also expressed in *C. sinensis* and *P. trifoliata* libraries infected with *Citrus leprosis virus* (CiLV) and *Citrus tristeza virus* (CTV), respectively. *P. trifoliata* is resistant to CTV and therefore the expression of LOX in this interaction was expected.

Several fruit ESTs associated with LOX were also identified within the CitEST database. The role of LOX in fruit development has been well documented (Griffiths *et al.*, 1999; Chen *et al.*, 2004a). LOXs play an important role in the production of volatile molecules that can influence flavor and aroma of many plant products (Robinson *et al.*, 1995). LOXs can also alter shelf life and organoleptical and nutritional characteristics of plant products (Robinson *et al.*, 1995). These are important features for the citrus industry, since fruit quality is essential for juice production as well as for the commercialization of fresh fruits.

Three *C. limonia* EST sequences related to LOX were also expressed during water stress. Membranes are main targets of degradative processes induced by drought and lipid peroxidation is one of the most important causes of cell deterioration, resulting in changes in the composition of fatty acids that affect the structural and functional properties of cell membranes (Smirnoff, 1993; Asada, 1999). The expression of LOXs during drought has been reported (Gigon *et al.*, 2004; Sofo *et al.*, 2004); however, little is known about the relationship between drought stress and LOX activity. Further studies are necessary to better understand the function of citrus LOXs in drought response.

Phospholipases

Phospholipases (PLs) in plants are known to be involved in several cellular responses, including plant growth, development, and responses to stress and defense. PLs play a pivotal role in the plant response via lipids pathway. The superfamily is involved in processes such as cytoskeletal rearrangement, membrane trafficking and degradation (Meijer and Munnik, 2003; Wang, 2004). Several studies have associated PL activity to stress response and defense, and the involvement of PLs and other enzymes in cell death has also been reported (Spiteller, 2003; Wang, 2005).

Plant PLs are classified according to their site of hydrolysis on phospholipids as phospholipase A_1 (PLA_1), phospholipase A_2 (PLA_2), phospholipase C (PLC), and phospholipase D (PLD).

The analysis within the CitEST database revealed sequences related to PLs. The assembling of the reads (E-value < -10) provided 9 contigs and 12 singletons with E-value < -10. The best hit in a BLASTX search as well as the homologous organism are shown in Table S1.

The phospholipase A (PLA) activity increased rapidly and systemically in response to wounding by attack of herbivores in leaves of tomato plants (Narvaez-Vasquez *et al.*, 1999). Multiple secretory-phospholipase A_2 ($sPLA_2$) genes have been also identified in plants (Lee *et al.*, 2005) and within the CitEST database, four reads were identified with high similarity to PLA_2. The clusterization provided only one contig that was confirmed to be 75% identical to a rice PLA_2 form, and this contig was formed by reads from healthy *C. sinensis* and *C. aurantifolia* libraries. This PLA_2 contig contains twelve conserved cysteine residues and sequences that are likely to represent the Ca(2+)-binding loop and active-site motif, which are characteristic of animal PLA_2s. In the CitEST project, the PLA_2 identified was sequenced from bark tissue, probably indicating a secretory possibility and a defense response in plant to a variety of stimuli including biotic and abiotic stresses (Lee *et al.*, 2005).

Under the control of cell surface receptors, the phosphoinositide-specific PLC isozymes hydrolyze the highly phosphorylated lipid phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$], generating two intracellular products: inositol 1,4,5-trisphosphate ($InsP_3$), which is an universal calcium-mobilizing second messenger; and diacylglycerol, which is an activator of protein kinase C (Rebecchi and Pentyala, 2000). Evidence suggests that $InsP_3$ may be involved in the signal transduction pathway for the development of the HR of *C. lemon* against *A. alternata* (Ortega and Perez, 2001). A search within the CitEST identified reads homologous to PLC genes. Clustering of these reads resulted in five contigs and nine singletons. The similarity among the deduced PLC proteins found ranged from 59% to 73% identity to PLC from *A. thaliana*, *O. sativa*, *Nicotiana rustica,* and *Glycine max*. The most representative group for PLC was contig 1, which was formed by reads from the libraries, bark and leaves of healthy *C. sinensis* and from *C. sinensis X. fastidiosa*-infected leaves.

PLD mediates the hydrolysis of membrane lipids and diacylglycerol-kinase-mediated phosphorylation of diacylglycerol (Wang, 2002). The expression of several PLD genes has been extensively reported in plant defense induced by the elicitor treatment as well as pathogen infection (Laxalt *et al.*, 2001; De Torres *et al.*, 2002). The PLD group was present in 14 reads clustered into three contigs within CitEST. The PLD genes identified were classified as different isoforms related to the *A. thaliana* (AAP42742.1) PLD protein. The CitEST PLDs were 80 to 83% identical to their orthologues. The largest PLD was also formed by reads from healthy *C. sinensis*.

Analysis of the CitEST database revealed the presence of several citrus PLs; however, since most contigs and singletons are incomplete, it was not possible to unequivocally classify them within the family. Further studies are necessary to better understand the genetic and biochemical heterogeneity of PLs and to relate the function of citrus PLs in HR.

Glutathione transferases and Glutathione peroxidases

Plant tissues contain several ROS scavenging enzymes (like glutathione peroxidase) and detoxifying lipid peroxidation products (like glutathione S-transferases and phospholipid-hydroperoxide glutathione peroxidase) (Chen *et al.*, 2004b), which protect cells from ROS and damage from stress conditions.

By using an *in-silico* analysis of the CitEST database, we have identified 36 contigs and four singletons related to glutathione transferase (GST) genes (Table S1). Taking into consideration that the CitEST database was constructed based on a number of ESTs produced during different stages of the development and challenges, it is obvious that, as seen in other plant species (Sappl *et al.*, 2004), several mechanisms are acting to induce citrus GSTs.

By combining keyword and BLASTX searching within the CitEST database with the sequence from public database that showed the best match with citrus sequences, we found full-length genes with high homology to GST 22 from soybean (McGonigle *et al.*, 2000). Whether this putative gene plays a role in stress tolerance remains to be experimentally demonstrated in plant species, including citrus. In addition, we found full-length copies of LeGST-T3, a GST protein from tomato that was found to protect yeast cells from H_2O_2-induced oxidative stress (Kilili *et al.*, 2004). This indicates that the putative citrus genes could play a role in cell protection from pro-oxidant-induced cell death.

Glutathione peroxidases (GPX) are enzymes that reduce organic hydroperoxides produced during oxidative stress. The over-expression of a GPX inhibited oxidative

stress - induced PCD in tomato (Chen *et al.*, 2004b). It is strongly suggested that specific GPXs, such as Phospholipid Hydroperoxide GPX (PHGPX), play a specific role in ROS; however, their specific role in plants remains to be elucidated (Milla *et al.*, 2003; Chen *et al.*, 2004b). PHGPX functions, in the removal of phospholipid hydroperoxides, are generated as products of lipoxygenase catalysed oxygenation of fatty acids (Ursini *et al.*, 1985).

The analysis of the CitEST database also revealed sequences related to GPXs, which formed 10 contigs and 2 singletons (Table S1). The analysis of these sequences shows GPX and PHGPX similar to the ones found in orange, pea, *Momordica charantia,* chickpea, and *Arabidopsis*.

At least two copies of a gene found in this study code for the PHGPX that was experimentally found to be responsible for salt-stress signaling in citrus (Holland *et al.*, 1993; Avsian-Kretchmer *et al.*, 2004).

As compared to other stress-related genes found in this broad-scale search, an observation that emerged was the proportion of genes that code for GPX in citrus. On the basis of these results, it is suggested that GPX is an enzyme that shows a low number of isoforms and/or copies in the genome of citrus and, probably, plays a role in a condition not strongly induced in this study.

Pathogenesis-related proteins

The induction of PR proteins is triggered stereotypically in plants by infection with fungal, bacterial, and viral pathogens, and is part of a more general response to biotic and abiotic stress (Datta and Muthukrishnan, 1999). PR proteins comprise a group of 17 protein classes (Christensen *et al.*, 2002), most of which present antifungal activity, such as the PR-3, PR-4, PR-8 and PR-11 proteins (also known as chitinases) and the PR-5 proteins (also known as osmotins, permatins or thaumatins).

The chitinases, which are vacuolar PR proteins, act in concert to defend against a specific class of pathogens, fungi (Wirdnam *et al.*, 2004). Moreover, there is also an indication that chitinases may act as lysozymes, by degrading bacterial cell walls (Tiffin, 2004). This class of proteins can be induced by ethylene treatment and in association with HR (Leubner-Metzger and Meins, 1999).

The analysis of the CitEST database revealed several reads related to chitinases, which formed 29 contigs and 24 singletons (Table S1). The analysis of these sequences shows various acidic classes I and II chitinases that match with citrus libraries from elsewhere. In addition, we found a number of sequences that relate to chitinase, in general, from other plant species such as *A. thaliana*, *Tulipa bakeri*, *Hevea brasiliensis*, *Gossypium hirsutum*, and *N. tabacum*. In rough lemon (*C. jambhiri*), the expression of the two acidic chitinases (I and II) was not constitutive and accumulated in leaves two hours after induction by wounding or inoculation with non-pathogenic isolates of *A. alternata* (Gomi *et al.*, 2002a). Our results reveal that a number of acidic class I and II chitinases are expressed in citrus. However, it remains unclear whether the citrus chitinases found in our study refer to isoforms of the same gene.

The PR-5 proteins are expressed in response to abscisic acid, ethylene, auxin, salinity, lack of water, cold, UV light, wounding, viral, fungal and oomycetal infection (Nelson *et al.*, 1992; Zhu *et al.*, 1995; Sato *et al.*, 1996). Within the CiEST database, we found 63 unique reads related to PR-5-like proteins, clustered into 10 contigs and four singletons (Table S1). These singletons encode a 219-amino acid ORF homologous to a sunflower (*H. annuus*) PR-5-like protein, a 190-amino acid ORF homologous to a turnip (*B. rapa*) PR-5-like protein, as well as a 182- and a 280-amino acid ORF, both homologous to a tobacco (*N. tabacum*) PR-5-like protein (Table S1). These singletons were originated from fruit libraries, from CTV-infected leaf libraries or from *X. fastidiosa*-infected leaf libraries, either from *C. reticulata*, *C. sinensis* or from *P. trifoliata* origin. The 10 PR-5-like contigs were assembled from two up to 13 reads each, mainly from the fruit libraries, but also from seed, bark, and flower libraries. Despite the fact that PR-5 proteins are expressed also in response to biotic stress, there were only five reads composing contigs which were originated from leaves infected either with *X. fastidiosa* or with CTV. Moreover, the majority of the PR-5-like reads forming contigs were from *C. sinensis* origin, secondarily from *C. reticulata* or *P. trifoliata* and seldomly from *C. aurantifolia* or *C. aurantium*. Two contigs homologous to tomato PR-5-like proteins, one homologous to an *Arabidopsis* PR-5-like protein (accession NP_173261), one homologous to a cultivated apple PR-5-like protein and one homologous to a grape PR-5-like protein presented complete coding sequences when aligned with the best hit in BLASTX search. As expected, a CitEST contig homologous to a *C. sinensis* PR-5-like protein (accession AAC02549; Table S1) presented high amino acid identity (97%). The 14 PR-5-like clusters found within the CitEST database, *i.e.*, 10 contigs and the four singletons, were considerably similar (from 73% to 97% similarity) to their corresponding most homologous amino acid sequences (Table S1).

To conclude, the CitEST database is considerably representative of citrus PR-5-like sequences, expressed in various biological situations, tissues and citrus species. Unexpectedly, within the CitEST database, no PR-5-like sequences associated with drought stress or with infection by *Phytophthora parasitica* were found. Surprisingly, there were only seven reads found, out of 63 reads in total, which originated either from *X. fastidiosa* infected-leaves or from CTV infected-leaves. Although most of the PR-5-like reads found within the CitEST database are associated with abiotic stress and plant development processes, these sequences potentially encode PR-5 proteins involved also in citrus HR. Therefore, the possible role of the CitEST PR-

5-like clusters in HR must be demonstrated experimentally. Further investigation is also necessary to clone complete citrus PR-5-like sequences, to validate their biological function and to better characterize their expression patterns under biotic and abiotic stress conditions.

Concluding Remarks

Despite a large number of potential pathogens that attempt to infect cultivated plants, disease is not the rule. Plants have an arsenal of mechanisms to defend themselves against pathogen invasion. One of these strategies is the expression of HR genes, elicited when resistant hosts are infected by incompatible races of a pathogen. It is characterized by localized cell death at the site of attempted infection, generating a physical barrier that restricts nutrient availability to the pathogen and prevents the dispersion of the pathogen to uninfected tissues. HR has been investigated extensively, and the increase of genomic sequences available has made it possible to indicate the existence of homologous genes in all plant species by *in silico* analysis. Moreover, the use of tools to search for sequence similarities at the amino acid level can reveal the overall patterns of gene network related to HR responses. In the present work, we tried to uncover the pathways that may lead to HR in citrus, based on the CitEST database. We found putative deduced proteins that can be important for HR and thus we have shown the putative pathways involved in this process. However, further studies are necessary to confirm the functions of these proteins and to better understand the genetic and biochemical network in citrus HR. To our knowledge, this is the first genome study that attempts to identify genes involved in HR in citrus plants. Functional characterization of them may contribute for the development of molecular tools to overcome economically important citrus diseases.

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Internet Resources

CitEST (Citrus ESTs database), http://biotecnologia.centrodecitricultura.br (June 25, 2006).

ClustalW program, http://www.ebi.ac.uk/clustalw/ (November 13, 2006).

NCBI, http://www.ncbi.nlm.nih.gov/ (November 6, 2006).

Supplementary Material

The following online material is available for this article:

Table S1

This material is available as part of the online article from http://www.scielo.br/gmb.

Associate Editor: Raquel Luciana Boscariol-Camargo

Table S1 - Description of citrus ESTs enconding proteins involved in HR.

Gene product and organism	Accession Number	Best *e*-value	Best Similarity	Number of reads	Number Contigs*	Number Singletons
Calcium channels						
two-pore calcium channel[*A. thaliana*]	gi|14349162	0.0	371/432	10	1 (C1)	0
two-pore calcium channel [*N. tabacum*]	gi|46275794	9e-52	133/170	5	1 (C2)	2
Chalcone synthase						
chalcone synthase [*C. sinensis*]	BAA81664	0.0	391/391	146	3 (C1; C3; C4)	8
chalcone synthase [*Betula pendula*]	CAA71904	2e-40	89/108	3	1	1
chalcone synthase [*Aubrieta deltoidea*]	AAG43406	1e-25	86/146	1	0	1
chalcone synthase 3 [*Ruta graveolens*]	Q9FSB7	3e-17	106/239	1	0	1
chalcone synthase [*Chrysanthemum indicum*]	AAM46963	7e-19	43/46	1	0	1
Chitinase						
Acidic class II chitinase [*C. jambhiri*]	BAC20285	e-169	282/293	185	6	11
Acidic class I chitinase [*C. jambhiri*]	BAC20284	e-177	289/300	154	4	6
Bulb chitinase-1 [*Tulipa bakeri*]	BAA88408	e-110	230/279	7	1	0

Chitinase [*C. sinensis*]	CAA93847	e-178	292/292	64	3	1
Chitinase [*Hevea brasiliensis*]	CAA09110	e-139	269/302	6	1	0
Chitinase CHI1 [*C. sinensis*]	AAC35981	4e-75	166/232	13	4	1
Chitinase-like protein [*G.hirsutum*]	AAQ56598	e-164	291/322	18	3	2
Chitinase/lysozyme [*N.tabacum*]	CAA55128	4e-48	126/195	4	1	2
Putative chitinase [*A. thaliana*]	CAA19698	9e-45	145/220	3	1	1
Putative class I chitinase [*A. thaliana*]	AAG48821	e-149	277/321	115	3	0
Receptor-like kinase CHRK1 [*N. tabacum*]	AAD52097	6e-76	299/484	9	2	0
Chorismate mutase						
putative chorismate mutase [*Fagus sylvatica*]	ABA54871	8e-122	265/326	7	1 (C5)	0
putative chorismate mutase [*A. thaliana*]	AAC73018	5e-100	219/290	4	1 (C4)	1
chorismate mutase ATCM2 [*A. thaliana*].	NP_196648	4e-65	145/182	2	1	0
chorimate mutase [*L. esculentum*]	AAD48923	4e-15	49/60	1	0	1
chorismate mutase-T [*A. thaliana*]	BAB10786	1e-35	97/137	1	0	1
chorismate mutase [*A. thaliana*]	AAM61395	3e-107	270/370	10	2 (C6; C7)	1
Cinnamic acid 4-hydroxylase						
cinnamate 4-hydroxylase CYP73 [*C. sinensis*]	AAF66066	0.0	485/485	59	2 (C1; C3)	2
Cinnamic acid 4-hydroxylase [*M.*	P37114	4e-74	108/121	2	1 (C2)	0

sativa]

Glutathione peroxidases						
Phospholipid hydroperoxide glutathione peroxidase [*C. sinensis*]	CAE46896	1e-91	167/167	13	1	0
CIT-SAP GPX4_CITS Probable phospholipid hydroperoxide glutathione peroxidase (PHGPx) (Salt-associated protein) [*C. sinensis*]	CAA47018	9e-92	167/167	49	1	2
Phospholipid glutathione peroxidase [*Pisum sativum*]	CAA04142	2e-89	193/246	28	2	0
Phospholipid hydroperoxide glutathione peroxidase [*Momordica charantia*]	AAL40914	4e-76	148/164	14	2	0
Putative phospholipid hydroperoxide glutathione peroxidase [*Cicer arietinum*]	CAD31839	8e-24	83/137	3	1	0
Glutathione peroxidase, putative [*A. thaliana*]	NP_564813	2e-69	139/153	5	1	0
Putative glutathione peroxidase [*A. thaliana*]	AAM67012	5e-68	145/169	8	1	0
Glutathione peroxidase, putative [*A. thaliana*]	NP_194915	6e-86	168/184	6	1	0

Glutathione transferases						
Glutathione S-transferase GST 22 [*G. max*]	AAG34812	3e-89	181/209	51	6	1
Glutathione S-transferase GST 14 [*G. max*]	AAG34804	2e-66	145/220	29	3	1
Glutathione S-transferase GST 23 [*G. max*]	AAG34813	5e-35	79/85	2	1	0
Glutathione S-transferase T3 LeGST-T3 [*L. esculentum*]	AAG16758	4e-82	173/223	29	5	1
Glutathione S-transferase [*Pisum sativum*]	BAC81649	8e-97	196/236	10	3	0
Glutathione transferase [*Carica papaya*]	CAA04391	2e-82	159/180	6	2	0
Glutathione S-transferase [*Euphorbia esula*]	AAF65767	3e-79	166/212	45	2	0
Glutathione S-transferase [*E. esula*]	AAF72197	6e-89	188/210	15	1	0
Glutathione S-transferase 2 [*Papaver somniferum*]	AAF22518	2e-85	175/215	37	2	0
Glutathione S-transferase/peroxidase [*Capsicum chinense*]	CAI48072	e-100	193/220	13	2	0
Glutathione S-transferase [*Thlaspi caerulescens*]	AY917145	5e-174	204/240	13	1	0
Glutathione transferase AtGST 10	BAB09723	4e-99	208/237	3	1	1

[*A. thaliana*]						
Glutathione S-transferase [*A. thaliana*]	AAG30140	6e-77	164/215	6	1	0
Putative glutathione S-transferase [*A. thaliana*]	AAM64426	5e-62	153/205	5	1	0
Putative glutathione S-transferase [*O. sativa*]	XP_470193	3e-71	163/218	14	1	0
Putative glutathione transferase [*O. sativa*]	BAD28950	7e-22	52/60	2	1	0
Glutathione S-transferase GST 9 [*Zea mays*]	AAG34817	3e-31	85/110	5	1	0
Putative glutathione S-transferase [*Phaseolus acutifolius*]	AAM34480	4e-13	42/48	2	1	0
Glutathione S-transferase [*Cucurbita maxima*]	BAC21263	5e-66	153/189	4	1	0
Isochorismate synthase						
isochorismate synthases, putative [*M. truncatula*].	ABE91019	1e-81	185/202	4	1 (C2)	0
isochorismate synthase [*C. annuum*]	AAW66457	3e-65	175/244	2	1	0
Isochorismate synthase, chloroplast precursor [*Catharanthus roseus*]	Q9ZPC0	1e-58	163/246	1	0	1
Lipoxygenase						
Lipoxygenase [Fragaria x ananassa]	CAE17327	e-126	219/255	4	1	1

Lipoxygenase [*Corylus avellana*]	CAD10740	4e-76	158/187	1	0	1
Lipoxygenase [*Solanum tuberosum*]	CAA65268	0.0	622/803	29	3	0
Lipoxygenase [*C. jambhiri*]	BAB84352	0.0	762/904	226	6 (C11)	10
Lipoxygenase 1 [*Sesbania rostrata*]	CAC43237	0.0	803/950	9	1 (C7)	0
Lipoxygenase 1 [*Prunus dulcis*]	CAD10779	8e-05	44/74	2	0	2
Lipoxygenase 2 [*Zantedeschia aethiopica*]	AAG18376	0.0	690/819	27	2 (C3)	0
Lipoxygenase 2 [*Nicotiana attenuata*]	AAP83137	4e-83	207/289	25	3	4
Metacaspases						
metacaspase 1 [*A. thaliana*]	AAP84706	e-104	202/243	9	2	0
metacaspase 3 [*A. thaliana*]	AAP44516	6e-38	125/226	1	0	1
metacaspase 7 [*A. thaliana*]	AAP84710	4e-88	206/267	5	1	0
metacaspase 1 [*L. esculentum*]	AAM51555	3e-84	181/234	2	0	2
putative metacaspase [*O. sativa*]	AAR06365	4e-20	81/150	1	0	1
NADPH						
NADPH oxidase [*N. tabacum*]	AAM28891	0.0	639/720	16	1 (C1)	0
FRO2-like protein; NADPH oxidase-like [*A. thaliana*]	BAA98161	e-104	235/310	4	1 (C2)	0
ferric reductase-like transmembrane component family protein [*A. thaliana*]	NP_199785	e-152	317/401	5	1 (C3)	1
FRO2 homolog [*A. thaliana*]	BAB08722	1e-79	178/272	1	0	1

respiratory burst oxidase homolog [*N. benthamiana*]	BAC56865	3e-69	81/119	1	0	1
Nitrate reductase						
nitrate reductase [NADH] (NR) [Petunia x hybrida]	P36859	0.0	495/567	11	1 (C10)	0
nitrate reductase [*Tilia platyphyllos*]	AAN15927	2e-152	294/337	3	1 (C11)	0
nitrate reductase [*Ricinus communis*]	AAG30576	1e-85	179/211	6	1 (C7)	0
nitrate reductase [*Brassica napus*]	BAA07395	4e-23	60/89	1	0	1
PR-5-like protein (Osmotin, Permeatin or Thaumatin)						
PR-5-like protein [*A. thaliana*]	AAM44961	1e-103	218/296	2	1	0
PR-5-like protein [*A. thaliana*]	NP_173261	1e-109	208/243	13	1	0
PR-5-like protein [*A. thaliana*]	BAB11214	5e-75	158/203	3	1	0
PR-5-like protein [*A. thaliana*]	AAM64698	1e-101	193/231	8	1	0
PR-5-like protein [*Citrus sinensis*]	AAC02549	2e-63	111/114	3	1	0
PR-5-like protein OLP1 [*L. esculentum*]	Q41350	1e-117	204/235	25	3	0
PR-5-like protein Mdtl1 [Malus x domestica]	AAC36740	4e-98	191/235	3	1	0
PR-5-like proteinVVTL1 [*Vitis vinifera*]	AAB61590	9e-89	177/227	2	1	0
PR-5-like protein [*Brassica rapa*]	T14428	6e-75	152/188	1	0	1
PR-5-like protein [*Helianthus*	AAM21199	9e-84	150/188	1	0	1

annuus]						
PR-5-like protein SE39b [*N. tabacum*]	BAA74546	1e-83	161/210	2	0	2
Peroxidases						
thioredoxin peroxidase [*N. tabacum*]	CAC84143	e-113	234/278	45	3	2
apoplastic anionic guaiacol peroxidase [*G. hirsutum*]	AAL92037	e-129	282/352	47	4	1
peroxidase [*Spinacia oleracea*]	CAA71492	e-111	246/314	23	2	0
ascorbate peroxidase [*Ipomoea batatas*]	AAP42501	e-126	231/250	41	4	0
peroxidase [*N. tabacum*]	AAK52084	e-125	271/354	16	1	0
peroxidase precursor [*Quercus suber*]	AAR31106	e-92	189/218	12	1	0
putative peroxidase [*A. thaliana*]	AAO50583	e-117	233/250	20	1	0
cationic peroxidase isozyme 40K precursor [*N. tabacum*]	BAA07664	e-98	240/321	53	7	0
class III peroxidase [*G. hirsutum*]	AAP76387	e-152	291/318	11	2	0
putative peroxidase [*A. thaliana*]	AAM20043	e-129	254/294	28	1	0
bacterial-induced peroxidase precursor [*G. hirsutum*]	AAD43561	e-136	272/320	22	1	1
peroxidase [*Populus balsamifera subsp. Trichocarpa*]	CAA66037	e-120	258/343	13	3	0
peroxidase ATP2a [*A. thaliana*]	CAA66863	e-126	244/281	21	2	0
putative L-ascorbate peroxidase [*A.*	BAC42431	9e-53	133/170	64	1	1

thaliana]						
peroxidase precursor [*G. max*]	AAD11482	e-127	267/316	10	1	1
putative ascorbate peroxidase [*A. thaliana*]	AAF23294	6e-98	183/202	7	1	0
putative trypanothione-dependent peroxidase [*O. sativa* (japonica cultivar-group)]	AAP50932	1e-86	248/406	3	1 (C22)	0
L-ascorbate peroxidase 1b (APX1b) [*A. thaliana*]	NP_187575	e-125	230/248	21	1	0
ascorbate peroxidase pir||T09845 L-ascorbate peroxidase (EC 1.11.1.11), glyoxysomal - upland cotton	AAB52954	e-119	217/223	3	1 (C65)	0
secretory peroxidase [*N. tabacum*]	AAD33072	e-133	246/257	16	1	5
chloroplast stromal ascorbate peroxidase [*Vigna unguiculata*]	AAS55853	e-165	321/369	10	1	0
Peroxidase pir||T10790 peroxidase (EC 1.11.1.7) - upland cotton	AAA99868	e-168	314/332	13	1	4
At2g29670/T27A16.23 [*A. thaliana*]	AAN18208	6e-88	212/278	13	1	0
thylakoid-bound ascorbate peroxidase [*N. tabacum*]	BAA78552	e-100	218/264	4	1	0
peroxidase precursor [*Populus kitakamiensis*]	BAA07240	e-127	263/327	7	1	0
putative ascorbate peroxidase	CAB64343	e-138	297/347	5	2	0

(TL29) [*L. esculentum*]						
putative peroxidase [*A. thaliana*]	AAP40436	5e-91	181/198	4	2	0
putative peroxidase ATP2a [*A. thaliana*]	AAM65003	3e-31	71/80	6	2	1
peroxidase ATP17a like protein [*A. thaliana*]	BAD44575	1e-17	51/67	2	1	0
peroxidase [*P. nigra*]	BAA11853	e-120	253/325	2	1	0
peroxidase-like protein [*A. thaliana*]	BAB08730	1e-25	62/67	1	0	1
peroxisomal ascorbate peroxidase [*Cucurbita* cv. Kurokawa Amakuri]	BAB64351	1e-18	52/66	1	0	1
glutathione peroxidase, putative [*A. thaliana*]	NP_564813	1e-15	41/43	1	0	1
peroxidase C2 precursor-like protein [*A. thaliana*]	AAN15499	3e-14	42/75	1	0	1
glutathione transferase [*Carica papaya*]	CAA04391	8e-62	130/160	1	0	1
korean-radish isoperoxidase [*Raphanus sativus*]	CAA62597	1e-42	130/233	1	0	1
putative glutathione peroxidase [*A. thaliana*]	AAL34198	4e-42	112/174	1	0	1
peroxidase [*Linum usitatissimum*]	AAB02926	1e-72	162/213	1	0	1
putative peroxidase [*A. thaliana*]	CAB39666	3e-20	61/150	1	0	1
glutathione peroxidase (EC	S33618	5e-30	42/44	1	0	1

1.11.1.9) - sweet orange						
ascorbate peroxidase [*Pimpinella brachycarpa*]	AAF22246	e-101	196/250	1	0	1
PER7_ARATH Peroxidase 7 precursor (Atperox P7)	Q9SY33	4e-58	149/214	1	0	1
cytosolic ascorbate peroxidase [Fragaria x ananassa]	AAB94574	2e-28	54/98	1	0	1
putative thioredoxin peroxidase [*O. sativa* (japonica cultivar-group)]	BAD28826	2e-26	64/73	1	0	1
peroxidase ATP1a [*A. thaliana*]	CAA66862	2e-29	112/199	1	0	1
peroxidase (EC 1.11.1.7), anionic, precursor - wood tobacco	B56555	3e-59	139/190	1	0	1
peroxidase [*A. thaliana*]	BAB10239	e-70	176/260	1	0	1
thylakoid-bound L-ascorbate peroxidase precursor [*Mesembryanthemum crystallinum*]	AAC19393	e-34	70/94	1	0	1
thylakoid-bound ascorbate peroxidase [*Cucurbita* cv. Kurokawa Amakuri]	BAA12029	e-60	130/146	1	0	1
thioredoxin peroxidase [*Secale cereale*]	AAC78473	4e-19	71/142	1	0	1
peroxidase [*A. thaliana*]	CAA66957	2e-43	89/140	1	0	1
putative peroxidase [*O. sativa* (japonica cultivar-group)]	BAB39281	2e-63	150/216	1	0	1

ascorbate peroxidase [*O. sativa* (japonica cultivar-group)]	BAB17666	2e-22	67/142	1	0	1
putative ascorbate peroxidase APX4 [*A. thaliana*]	AAP72143	3e-30	67/75	1	0	1
peroxidase [*N. tabacum*]	BAA82306	8e-71	155/235	1	0	1
PE48_ARATH Putative Peroxidase 48 (Atperox P48)	O81755	6e-20	61/77	1	0	1
Phenylalanine ammonia-lyase						
phenylalanine-ammonia lyase [*C. clementina x C. reticulata*]	CAB42794	0.0	708/713	69	1 (C3)	2
phenylalanine ammonia-lyase [*C. limon*]	Q42667	0.0	715/722	23	1 (C8)	3
phenylalanine ammonia-lyase [*Jatropha curcas*]	ABI33979	0.0	356/380	4	1 (C9)	0
phenylalanine ammonia-lyase [*Nerium oleander*]	AAX18625	2e-107	200/208	2	1 (C10)	0
phenylalanine ammonia-lyase 1 [*Manihot esculenta*]	AAK62030	e-116	231/270	1	0	1
phenylalanine ammonia-lyase [*Populus trichocarpa*]	P45730	9e-55	110/145	1	0	1
Phospholipases						
putative phospholipase A2 [*O. sativa*]	XP_468549	6e-47	98/120	4	1	0
phosphoinositide-specific	AAB03258	0.0	512/602	20	1	0

phospholipase C P13 [*G. max*]						
putative phospholipase C [*A. thaliana*]	AAC32238	e-127	229/276	2	1	0
phospholipase C [*O. sativa*]	CAC81703	e-101	94/118	4	0	4
phospholipase C [*A. thaliana*]	NP_175685	e-108	200/222	1	0	1
1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase [*N. rustica*]	CAA72681	e-147	273/306	1	1	0
phosphoinositide-specific phospholipase C P12 [*G. max*]	gi|7435171	5e-33	67/85	1	0	1
phosphoinositide-specific phospholipase C [*N. rustica*]	CAA65127	e-112	236/286	3	2	0
PLC [*A. thaliana*]	AAL16172	9e-92	178/204	1	0	1
ATPLC2 phospholipase C (ATPLC2) [*A. thaliana*]	NP_187464	2e-70	153/191	3	0	3
phospholipase C2 [*N. tabacum*]	AAF33824	3e-73	164/214	1	0	1
PLD [*A. thaliana*]	AAP42742	e-160	289/312	14	3	1
Potassium channels						
CNGC1 [*A. thaliana*]	NP_200125	0.0	366/386	48	11	7
CNGC2 [*G. hirsutum*]	AAX18166	0.0	337/359	19	3	0
CNGC3 [*A. thaliana*]	NP_566075	e-100	204/245	2	0	2
CNGC4/ HLM1 [*A. thaliana*]	Q94AS9	6e-96	175/194	1	0	1
CNGC9 [*A. thaliana*]	NP_194785	1e-81	179/234	1	0	1
CNGC10 [*A. thaliana*]	NP_563625	1e-12	83/160	2	1	0
CNGC14 [*A. thaliana*]	NP_850056	2e-84	121/158	1	0	1

CNGC15 [*A. thaliana*]	NP_180393	3e-60	135/182	2	0	2
CNGC17 [*A. thaliana*]	NP_194765	3e-179	347/394	5	1	2
CNGC19 [*A. thaliana*]	NP_188396	2e-118	254/311	6	1	0
Superoxide dismutase						
SODM_HEVBR Superoxide dismutase [Mn], mitochondrial precursor S39492 superoxide dismutase (EC 1.15.1.1) (Mn)	P35017	e-105	199/231	43	1	0
Fe-superoxide dismutase precursor [*M. sativa*]	AAL32441	e-105	216/260	58	3 (C4)	0
copper-zinc superoxide dismutase [*Nelumbo nucifera*]	AAW80437	2e-55	107/112	3	1	0
Fe-superoxide dismutase precursor [*A. thaliana*]	AAM61633	3e-86	177/205	3	1 (C3)	0
iron superoxide dismutase 3 [*A. thaliana*]	AAM63713	3e-64	139/178	4	1 (C7)	0
superoxidase dismutase [*L. esculentum*]	AAQ09007	3e-77	75/83	4	1(C9)	0
superoxide dismutase [Fe] [*L. esculentum*]	CAE22480	3e-76	179/252	2	1(C18)	0
Cu/Zn-superoxide dismutase copper chaperone precursor [*G. max*]	AAK01931	3e-99	199/217	7	2 (C16)	0
copper/zinc superoxide dismutase [*C. limon*]	AAQ14591	7e-85	151/152	30	4 (C19)	1

Cu/Zn superoxide dismutase [*C. sinensis*]	CAA03881	5e-67	125/126	59	4 (C12)	5
MnSOD [H. brasiliensis]	CAB53458	3e-16	40/48	1	0	1
manganese superoxide dismutase [*Capsicum annuum*]	AAB88870	1e-50	101/110	1	0	1
putative CuZn-superoxide dismutase [*Populus tremula* x *P. tremuloides*]	CAC33847	3e-69	116/127	2	0	2
iron superoxide dismutase 3 [*A. thaliana*]	AAC24834	2e-59	142/200	2	0	2
iron-superoxide dismutase [*G. max*]	AAQ13492	3e-17	69/120	1	0	1
copper/zinc superoxide dismutase [Bruguiera gymnorrhiza]	BAB78597	3e-13	38/40	1	0	1
superoxide dismutase (EC 1.15.1.1) (Mn) precursor - curled-leaved tobacco	S03639	e-62	128/153	1	0	1
iron superoxide dismutase 3 [*A. thaliana*]	BAB11186	2e-47	65/106	1	0	1

*Between parentheses are represented the contigs (C) commented in the Results and Discussion. As search for different proteins had been made in separate archives, different contigs (representing different proteins) can have the same number.