



Signaling pathways in a Citrus EST database

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Abstract

Citrus spp. are economically important crops, which in Brazil are grown mainly in the State of São Paulo. Citrus cultures are attacked by several pathogens, causing severe yield losses. In order to better understand this culture, the Millennium Project (IAC Cordeirópolis) was launched in order to sequence Citrus ESTs (expressed sequence tags) from different tissues, including leaf, bark, fruit, root and flower. Plants were submitted to biotic and abiotic stresses and investigated under different development stages (adult vs. juvenile). Several cDNA libraries were constructed and the sequences obtained formed the Citrus ESTs database with almost 200,000 sequences. Searches were performed in the Citrus database to investigate the presence of different signaling pathway components. Several of the genes involved in the signaling of sugar, calcium, cytokinin, plant hormones, inositol phosphate, MAPKinase and COP9 were found in the citrus genome and are discussed in this paper. The results obtained may indicate that similar mechanisms described in other plants, such as *Arabidopsis*, occur in citrus. Further experimental studies must be conducted in order to understand the different signaling pathways present.

Key words: Citrus, cell signaling, genomics.

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Introduction

Cell-cell and cell-environment communication (signal transduction) is crucial for the development of multicellular organisms. In plants, cell signaling connects the environmental input to the intracellular responses in plants. Cold, drought, and salt stresses all stimulate the accumulation of compatible osmolytes and antioxidants. Given the multiplicity of signals in a plant lifetime, there is a wide range of external and internal signals leading to cell response. Exogenous and endogenous signals play an important role in cell metabolism leading to growth and defense responses (Xiong *et al.*, 2002). Recent advances in plant signaling research revealed that plants respond to signals activating the cell signaling network that comprises calcium sensors and signaling, sugar signaling, plant hormone

response (ABA, JA), cytokinins, phosphorylation cascade and peptides which have well characterized roles in the multicellular coordination of plant physiology, development, defense and other processes. But little is known still about plant cell signaling, although the *Arabidopsis* and plant genomics allowed the identification of many signaling pathways (Button *et al.*, 2006).

The identification of all signaling components and messengers that mediate transduction pathways and the analysis of their function and regulation and cross talk among these components should help in understanding the inner workings of plant cell responses to diverse signals. Knowledge about cell signaling is also important for the continued development of rational breeding and genetic modification strategies to improve tolerance in crops. In the present work we attempt to present the citrus components and signaling pathways network grouped into different categories. We also indicate some possible features for the cross talk in citrus development and physiological processes.

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Calcium signaling pathway

Ca²⁺ is an important cell messenger with fundamental roles in signal transduction networks of all eukaryotes. In plants, Ca²⁺-dependent protein kinase (CDPK) activities are related to growth, reproduction and development, including responses to drought, cold and salt stresses, mechanical wounding and pathogens. Moreover, the Ca²⁺ signals are implicated in responses to hormones such as ABA, gibberellic acid, cytokinin and others.

The transient increases in cytosolic Ca²⁺ and ion influx through calcium channels are perceived by CDPKs and the SOS3 family (Xiong *et al.*, 2002). CDPKs (calcium-dependent protein kinases or calmodulin-like domain protein kinases) are activated by the binding of calcium to their calmodulin-like regulatory domains. The CDPKs superfamily consists of serine/threonine protein kinases with a C-terminal calmodulin-like domain with up to 4 EF-hand motifs that directly bind to Ca²⁺. The carboxyl terminal domains of CRKs (CDPK-related kinases) have sequence similarity to the regulatory domains of CDPKs, but do not bind calcium. PPCKs (PEP carboxylase kinases) contain only one catalytic domain. PRKs (PPCK-related kinases) have a carboxyl-terminal domain that has no similarity to that of any other member of the superfamily. CCaMKs (calcium and calmodulin regulated kinases) bind both calcium ions and the calcium/calmodulin complex, whereas CaMKs (calmodulin-dependent protein kinases) bind the calcium/calmodulin complex, but not calcium.

The structural similarity among CDPKs (also called CPKs) allowed their classification into three groups: CDPK, CCaML and CRK. Their unique structure is defined by a kinase catalytic domain in the C-terminal fused to a regulatory calmodulin-like domain (CaM-LD). Two models are in use representing the calcium activation mechanism (Harper *et al.*, 2004). In the first model, CDPKs represent sensor responders in which a flexible tether joins the kinase CaM-LD domain covalently. Upon calcium stimulation, this domain replaces the autoinhibitor segment and starts the signaling cascade. The second model proposes that the regulation by CRKs, CCaMKs and SnRK3 occurs through an exogenous Ca²⁺ binding that relays the conformational change to the kinase domain as a consequence of calcium binding.

Cytokinin signaling pathway

Cytokinins are important regulators of a large number of processes in plant development. The plasticity and adaptation allow plants to respond sensitively and quickly to their environmental stimuli. Recent studies have demonstrated that cytokinin signaling involves a multistep two-component signaling pathway through a common model of cytokinin signaling that is likely representative in plants. Each system consists of a sensor protein histidine kinase, which is anchored in the cell membrane, and a cytoplasmic

response regulator, whose activity is modulated by the sensor (Harper *et al.*, 2004).

The histidine kinases are named CKI1 (from cytokinin-independent 1 two-component response regulator), AHK2 (from *Arabidopsis thaliana* histidine kinase 2), AHK3 and CRE1 (from cytokinin receptor). Cytokinin-Independent 1 (CKI1) belongs to a group of putative plant histidine kinases whose members do not appear to act as ethylene receptors (Pischke *et al.*, 2006). The regulators are named AHPs (from 1 to 5, *Arabidopsis thaliana* histidine phototransmitter) and ARRs classified in A-type and B-type *Arabidopsis thaliana* response regulators (Oka *et al.*, 2002).

The cytokinin signal is perceived by histidine protein kinases at the plasma membrane: CKI1, AHK2, AHK3 or CRE1. Following the cytokinin signal, these histidine protein kinases initiate a signaling cascade through the phosphorelay that results in the nuclear translocation of AHPs from the cytosol. Activated AHPs interact with sequestered ARRs or ARR complexes in the nucleus, transfer the phosphate to the receiver domain of their cognate B-type ARRs, and in turn release the transcription activator ARRs from putative repressors in the nucleus. The dephosphorylated AHP shuttles back to the cytosol, where it can be rephosphorylated. The liberated ARRs bind to multiple cis elements in the promoters of target genes. The activation of the transcription repressor ARRs as cytokinin primary response genes provides a negative feedback mechanism (Oka *et al.*, 2002).

Ethylene signaling pathway

Ethylene is a plant hormone involved in several processes including flower and leaf senescence, fruit ripening and biotic/abiotic stress responses such as drought, chilling, flooding, wounding and pathogen infection. The main signaling components of the ethylene transduction pathway include (i) an ethylene receptor (ETR) to sense the hormone; (ii) the downstream constitutive triple response 1 (CTR1) Raf-like serine/threonine kinase; followed by (iii) the ethylene insensitive-2 (EIN2) positive regulator of the pathway; then (iv) the family of ethylene insensitive-3 (EIN3) transcription factors which regulate the expression of (v) the family of ethylene response element binding protein (EREBP) transcription factors (Guo and Ecker, 2004). The addition of ethylene inactivates the ethylene receptors (negative regulation of downstream responses), which in turn no longer activates CTR1, resulting in the release of suppression of EIN2 and consequential activation of EIN3. The EIN3 transcription factor binds to regulatory sequences in the promoter of ethylene-regulated genes initiating a transcriptional cascade that culminates in ethylene response.

Several EREBP transcription factors are known to be immediate targets of the EIN3 transcription factors. The EREBP factor named ethylene response factor 1 (ERF1) is

involved in both ethylene and jasmonate signaling, representing a cross-talk point between the two signal transduction pathways (Lorenzo *et al.*, 2003). Other EREBP factors regulate gene expression via association with the *cis*-element GCC-box present in several ethylene-responsive genes involved, for instance, in resistance to pathogens and differential cell growth.

The ethylene signal transduction pathway seems to be conserved among agronomically important dicot and monocot plants, though some particularities may be observed (Klee, 2004).

ABA signaling pathway

ABA (abscisic acid) is an important hormone associated to late seed development and adaptation to environmental stresses. A simple ABA signaling pathway has not been defined yet; however, some ABA insensitive genes have been identified, such as ABI1 and ABI2, which are involved in vegetative and seed ABA responsiveness. ABI1 and ABI2 genes encode homologous type 2C protein phosphatases and ABI3, ABI4 and ABI5 genes encode transcription factors of the B3 domain, APETALA2 (P2) domain and bZIP factor classes, respectively. Pei *et al.* (1998) have identified farnesyl transferase (ERA1) as an attenuator of seed and vegetative ABA sensitivity in mutants with enhanced response to ABA. It has been reported that ABI1/2 acts at or above ERA1 and both of these genes act at or above ABI3 and ABI5 (Pei *et al.*, 1998).

In *Arabidopsis thaliana*, several different genes have been associated with the ABA response including PGGT-I (Johnson *et al.*, 2005), the gene encoding the b-subunit of protein geranylgeranyltransferase type I (GGGT I). Cross-talk between ABA and Inositol 1,4,5-triphosphate dephosphorylation is also important in this signal transduction since mutation in *fryl1*, the inositol polyphosphate 1-phosphatase encoding gene, leads to super-induction of ABA- and stress-responsive genes (Xiong *et al.*, 2002). Other genes involved in the ABA response participate in the RNA metabolism like *hyl1* (Lu *et al.*, 2002), a gene encoding a double-stranded RNA binding protein that is related to activity of MAP kinases (Lu *et al.*, 2002); *abh1* (Hugouvieux *et al.*, 2001), a gene encoding an mRNA cap binding protein that modulates the ABA signaling by affecting transcription of early ABA signaling elements; and *sad1*, that encodes a polypeptide similar to multifunctional Sm-like snRNP proteins required for mRNA splicing, export and degradation (Xiong *et al.*, 2001). Other non-transcription factor-encoding genes are also involved in the ABA response such as genes encoding NADPH oxidase, *rboHD* and *F* (Kwak *et al.*, 2003), showing that reactive oxygen species are second messengers in ABA signaling.

Sugar signaling pathways

Soluble sugars, such as glucose and sucrose, seem to regulate diverse plant developmental, physiological and

metabolic processes through several pathways (Rolland *et al.*, 2002). Rolland *et al.*, (2002) have proposed a model for possible sugar signals and sensing sites. According to these authors, sugar such as glucose (Glc) and fructose (Fru) can be transported into the cell by hexose transporters. After hexokinase (HXK) catalyzed phosphorylation, Glc enters the metabolism. The HXK sugar sensor (a protein in the cytosol or in association with an organelle) could activate a signaling pathway through HXK interacting proteins or affect transcription directly after nuclear translocation. Rolland *et al.* (2002) also suggest that different HXK and fructokinase (FRK) isoforms and HXK-like proteins have distinct metabolic and signaling functions. Metabolic intermediates could trigger signal transduction by activating metabolite sensors. These authors propose that SnRK protein kinases might act as sensors of metabolic activity. SnRKs play an important role in carbon metabolism by directly phosphorylating and inactivating the biosynthetic key enzymes 3-hydroxy-3-methyl glutaryl CoA reductase, nitrate reductase (NR) and Suc phosphate synthase (Sugden *et al.*, 1999).

The yeast SNF1 Ser/Thr PK is well characterized and is one of the major components in sugar signaling. It has been demonstrated that low glucose concentrations activate SNF1 kinase, which result in the phosphorylation of the transcriptional repressor Mig1, causing its translocation to the cytoplasm and derepression of target genes (Alberti *et al.*, 2003). SNF1 can also directly affect the transcription machinery through the interaction with the Srb/mediator complex of RNA polymerase II and histone phosphorylation (Lo *et al.*, 2001). Molecular analyses have revealed the existence of a large family of SnRKs in plants and several SnRKs have been shown to complement the yeast *snf1?* phenotype (Hrabak *et al.*, 2003). The SNF1 kinase is a conserved complex and the subunits SNF4, SIP homologue and SnRK, also called SNF1-related kinase, have been reported in plants (Smeekens, 2000).

Jasmonic acid signaling pathway

Jasmonates (JAs) are signaling molecules that orchestrate plant responses to biotic and abiotic stresses locally and systemically. The term jasmonate includes the active intermediates in the jasmonic acid biosynthesis as well as the derivatives of jasmonic acid. JAs are widely distributed in plants and affect several processes such as fruit ripening, pollen maturation, root growth and defenses against insects and pathogens. It has been proposed that wounding causes release of linolenic acid, which is a precursor for JA, and that an E3 ubiquitin ligase probably regulates most JA responses in *Arabidopsis* (Turner *et al.*, 2002). JA signaling is best known in *Arabidopsis* and tomato; however, there are discrepancies in the proposed pathways and it is not clear if these divergences are due to differences in the mechanisms or lack of knowledge.

Two mechanisms by which JAs activate gene expression have been reported. The best characterized pathway involves components including coronatine insensitive 1 (COI1) and jasmonic acid resistant1 (JAR1) (Staswick *et al.*, 2002). Cyclopentenones, such as oxo-phytodienoic acid (OPDA) and the cyclopentanone JA participate in this signal transduction pathway. These compounds activate and repress the expression of several genes. The second mechanism involves only the cyclopentenone jasmonates, such as OPDA, which can alter gene expression (Farmer *et al.*, 2003).

Two multiprotein complexes have been reported to play a central role in jasmonate signaling; one is the COP9 signalosome (CNS), further discussed in this paper, and the other is the SCFCOI1 complex. The defining feature of the SCFCOI1 complex is COI1, which can associate physically with Skp-like proteins, cullin and *Arabidopsis thaliana* RING-box1 (AtRbx1) to form active SCFCOI1 complexes that function as E3-type ubiquitin ligases (Xu *et al.*, 2002). Once activated by jasmonates, SCFCOI1 targets regulatory proteins for ubiquitination by modifying their activity or by targeting their proteolysis. Histone deacetylase can interact with COI1 and is a newly identified candidate regulator of jasmonate responses (Devoto *et al.*, 2002). Orca3, an ap2/2 (AP2)/ethylene-responsive factor (ERF)-domain transcription factor is one of the regulatory proteins found downstream in the jasmonate signaling pathway (Meme-link *et al.*, 2001).

COP9

The COP9 signalosome (CSN) was first identified as an important photomorphogenesis actor in plants. Biochemical studies in both plants and animals have demonstrated that CSN is a conserved nuclear protein complex with eight subunits highly homologous to the lid sub-complex of 26S proteasome that participates in several cellular processes, ranging from transcriptional regulation to protein degradation. Protein kinases called CSN-associated kinases are involved in these cellular responses.

CSN activities mainly include the following: an associated kinase that phosphorylates p53, c-Jun and other regulatory proteins; deneddylation of the Cullin-1 subunit of the SCF E3 ubiquitin ligase complex; mediation of the nuclear export of p27kip1; and mediation of the nuclear import of COP1. One of the major targets is the SCF ubiquitin ligase complex that catalyzes a key step in ubiquitination. Also, it has been pointed out that the neddylation pathway is a target of CSN suggesting that this role has a significant influence over auxin response. However, the mechanisms and interconnections of COP9 functions are still not clearly understood (Wei and Deng, 2003).

Kinases and phosphatases

Phosphorylation and dephosphorylation are catalyzed by kinases and phosphatases, respectively, and many

signal transduction processes depend on the reversible phosphorylation of proteins.

MAPKs (mitogen-activated protein kinases) are serine/threonine protein kinases that play key roles in integrating multiple intracellular signals transmitted by various second messengers. All eukaryotes utilize MAPK cascades to convey signals that are generated from the perception of both extra- and intracellular stimuli. MAPK cascades are multicomponent pathways that consist of at least three protein kinases, mediating sequential phosphorylation reactions. A MAPK kinase kinase (MAPKKK) phosphorylates and activates a MAPK kinase (MAPKK), which, in turn, activates a MAPK by phosphorylation (Chen and Cobb, 2001). The cascades of the MAPKs are involved in ethylene signal transduction, JA biosynthesis pathway, phytoalexin biosynthesis in parsley cell cultures, defense responses and hypersensitive cell death (Ligterink and Hirt, 2001).

Glycogen synthase kinases (GSK) are a family of cytoplasmic kinases that belong to the mitogen-activated protein kinase superfamily and are found in animals, fungi, and plants (Tavares *et al.*, 2002). There is evidence that two *Arabidopsis* GSK3 are involved in floral development (Dornelas *et al.*, 2000) and one plays a crucial role in brassinosteroids signaling and in alfalfa GSK3 is involved in wound signaling (Jonak and Hirt, 2002).

Casein kinases are critical in cell division and differentiation across species. Liu *et al.* (2003) suggested that casein kinase 1 (Ck1) from rice might be involved in the root development signaling pathways that are regulated by abscisic acid and brassinosteroid hormones. On the other hand, casein kinase 2 (Ck2) is one of the most pleiotropic protein kinases with hundreds of protein substrates involved in a variety of cellular functions with special reference to signaling, nuclear organization, and gene expression (Boldyreff *et al.*, 1993).

Protein Ser/Thr phosphatases are divided into the protein phosphatase P (PPP) and protein phosphatase M (PPM) families, which have distinct amino acid sequences and crystal structures (Kutuzov and Andreeva, 2002). The PPM family mainly consists of PP2C phosphatases and the PPP family contains protein phosphatases 1 (PP1), 2A (PP2A), 2B (PP2B), 5 (PP5) and RdgC/protein phosphatase 7 (PP7) (Kerk *et al.*, 2002). In plants, one of the roles of PP2Cs is involved in the regulation of MAPK pathways (Meskiene *et al.*, 1998) and two PP2C from *Arabidopsis* (ABI1 and ABI2) act as negative regulators of ABA signaling (Merlot and Firtel, 2003).

Kinase-associated protein phosphatase (KAPP) interacts with many other plant receptor kinases. For example, KAPP is phosphorylated by CLV1 (CLAVATA1) and dephosphorylates the kinase domain of this receptor *in vitro* (Stone *et al.*, 1998).

Tyrosine-specific protein phosphatase have roles in processes as diverse as pollen development (Gupta *et al.*,

2002), stomatal opening, and regulate the mitogen-activated protein kinases (MAPKs) involved in a variety of signaling pathways.

Inositol phosphate

Inositol metabolism is essential for the development of plants, animals, and some microorganisms. Inositol is a sugar that plays essential roles in many cellular processes including membrane formation, cell wall biogenesis, stress response as well as signal transduction. It has been implicated in stress tolerance and possibly also in carbohydrate transport (Liu *et al.*, 2006). Inositol phosphates are essential to signaling in almost all organisms and in plants, inositol hexaphosphate provides for phosphate storage (Pera *et al.*, 2006).

Signaling peptides

Plant peptides are important in various signaling pathways and have been identified in several plants (for a review see Ryan *et al.*, 2002). The plant peptide systemin was discovered during a search for the systemic wound signal that regulates the expression of defensive genes in tomato leaves in response to insect attacks or other severe mechanical wounding (Chilley, 2003). Systemin is recognized by the SR160 receptor-like kinase, which induces defense gene activation. The plant peptide phytoalexin (PSK) interacts with the receptor-like kinase PSKR and activates a set of genes responsible for cellular dedifferentiation and redifferentiation. Clavata3 is translated, secreted and binds a Clavata1/Clavata2 receptor-like kinase complex, which regulates the balance between meristem cell proliferation and differentiation (Rojo *et al.*, 2002).

Materials and Methods

Citrus ESTs (expressed sequence tags) have been sequenced by the Millennium Project (IAC Cordeirópolis). Different tissues (leaf, bark, fruit, root and flower) from *Citrus spp.* and *Poncirus trifolia* were submitted to biotic and abiotic stresses and investigated at different development stages (adult vs. juvenile). Several cDNA libraries were constructed and the sequences obtained formed the Citrus ESTs (CitEST) database with almost 200,000 sequences.

To investigate signaling pathway components in the database (<http://citest.centrodecitricultura.br>), we used two strategies: the first one was a key word search and the second was a BLASTn or tBLASTn (Altschul *et al.*, 1997) search using well annotated queries retrieved in Genbank (www.ncbi.nlm.nih.gov). The parameters used for the reverse annotation were an e-value filter of e^{-4} and no low complexity filtering. Once selected, the reads were submitted to clustering by using the program CAP3 and the assembly results were organized by project/gene name or subject (Huang and Madan, 1999). Manual annotation confirmed ortholog similarity.

Results and Discussion

Calcium signaling pathway in *Citrus*

Ca^{2+} signals play an important role in many aspects of plant growth and development, including the response to biotic and abiotic stresses. One of the most intriguing aspects of Ca^{2+} signaling in plants is the occurrence of a large family of related isoforms, in contrast to a more specific situation in animals where, for example CaM isoform is encoded by three genes.

Advances over the last decade in genomics have made it possible to identify gene expression as well as orthologs by *in silico* search. The comparative approach for sequence similarities showed the overall patterns of gene network systems at the amino acid level. Bioinformatics in the CitEST database indicated a great number of contigs and singlets related to calcium signaling proteins (Table 1). The most abundant form of a calcium sensor found was CDPK1 including the forms CDPK2, CDPK3, CDPK6, CDPK7, CDPK9, CDPK19, and CDPK-like. For CRK and CaMK3 only one singlet was found for each gene, indicating that further investigation is needed. In contrast, the SNRK1 gene seemed to be highly expressed in the Citrus transcriptome in which several ESTs corresponding to isoforms of SNRK1 were identified (CIPK1, CIPK8, CIPK9, CIPK12 and CIPK25). The calcinerin family was identified through several isoforms, including CBL10 (the most abundantly expressed), CBL3, CBL1 and a calcinerin-like ortholog. The main calcium signaling components found in the CitEST database are shown in Figure 1A.

Our results are in agreement with other findings in plants. In *Arabidopsis*, 67 CDPKs implicated in Ca^{2+} signaling have been found (Harper *et al.*, 2004). Among this group, 34 were identified as CDPKs, 8 genes of CRK, 38 SNRKs and the largest family identified was SnRK3. In the Citrus EST database, the largest family was CDPKs followed by SNRKs.

Although the families of kinase have been implicated in calcium signaling through different mechanisms, they all bind calcium sensors via EF-hands domains. Calcium concentration oscillation relays plant cell responses to environmental stresses through a complex interconnected network. Many abiotic stimuli induce a transient cytosolic calcium increase and consequently, the gene expression of calcium sensors as CaMs and CDPKs are often induced.

The remaining questions regarding the possible mechanisms by which Ca^{2+} regulates diverse biochemical and molecular processes and eventually physiological processes in response to diverse signals are beginning to be understood. Our study suggests some common features between Citrus and other plants such as *Arabidopsis* and rice. Further experimental approaches, through microarray experiments for example, shall contribute to answers to these questions.

Table 1 - Ortholog genes of Calcium signaling pathways found in the Citrus EST database.

	Gene	Gene product	Organism	Accession #	Clusters	
					Contigs	Singlets
Calcium signaling pathways		CDPK	<i>Arabidopsis thaliana</i>	gb AAT06478.1	4	7
		CDPK1	<i>Cicer arietinum</i>	gb AAP72281.2	3	2
		CDPK2	<i>Cicer arietinum</i>	gb AAP72282.2	9	0
		CDPK3	<i>Oryza sativa</i>	gb AAN41657.1	2	0
	<i>CDPK¹</i>	CDPK6	<i>Triticum aestivum</i>	gi 47522360	0	1
		CDPK7	<i>Fragaria x ananassa</i>	AAB88537.1	0	4
		CDPK9	<i>Nicotiana tabacum</i>	gi 3283996	0	2
		CDPK19	<i>Arabidopsis thaliana</i>	NP_850853.1	1	0
		CDPK-like	<i>Solanum tuberosum</i>	gb AAM29184.1	1	2
	<i>CRK²</i>	CRK	<i>Arabidopsis thaliana</i>	gi 15226426	0	1
	<i>CaMK³</i>	CaMK3	<i>Arabidopsis thaliana</i>	gb AAD12016.1	2	0
		SNRK1	<i>Cucumis sativus</i> <i>Nicotiana tabacum</i>	CAA71142.1 gi 496385	0	2
		CIPK1	<i>Arabidopsis thaliana</i>	dbj BAB02040.1	4	0
	<i>SnRK⁴</i>	CIPK8	<i>Arabidopsis thaliana</i>	gi 7446447	0	1
		CIPK9	<i>Arabidopsis thaliana</i>	gb AAK26845.1	1	1
		CIPK12	<i>Arabidopsis thaliana</i>	gi 7446437	0	1
		CIPK25	<i>Arabidopsis thaliana</i>	gb AAL41008.1	1	0
		CBL10	<i>Arabidopsis thaliana</i>	gb AAO72364.1	2	0
	<i>CBL⁵</i>	CBL3	<i>Arabidopsis thaliana</i>	gb AAM91280.1	1	0
	CBL1	<i>Arabidopsis thaliana</i>	dbj BAC43389.1	1	0	
	Calcinerin-like	<i>Eucalyptus grandis</i>	AF197330_1	0	3	

Cytokinin signaling pathway in *Citrus*

A search for cytokinin members of the signaling pathway in the CitEST database indicated the pathways shown in Figure 1B. The most abundant members were AHK3, AHP5 and regulators ARR A and B-type. No full-length sequence was identified in the database and some genes such as CKI1 and CRE1 showed a weak similarity through Blastn search in CitEST.

Several contigs were related to AHK3 and ARR2 but few were assembled showing similarity to other members of the ARR family such as ARR6, ARR3, ARR11, ARR1 and ARR2 (Table 2). Considering these findings, one can suggest that the homologues of the three key proteins in a His/Asp phosphorelay are expressed in Citrus, as well as the response regulators. Those results are quite in agreement with the genes that have been identified in *Arabidopsis* and other eukaryotes such as yeasts (Pischke *et al.*, 2006).

The *Arabidopsis* genome has revealed three cytokinin receptors (CRE1 and its homologues AHK2 and AHK3). Similar components are also found in maize, suggesting a conservation of the cytokinin signaling mechanism in plants (Kiba *et al.*, 2005). The multistep two-component phosphorelay mechanism found in *Arabidopsis* is reminis-

cent of the bacterial two-component signaling system (Suzuki *et al.*, 2001).

In Citrus, we found evidence for the four major steps in the cytokinin signaling pathway: AHK sensing and signaling, AHP nuclear translocation, ARR transcription activation, and a negative feedback loop through cytokinin-inducible ARR gene products (Fig. 1B).

The importance of histidine protein kinase activity and phosphorelay has not been clearly demonstrated in plant cells yet. Nevertheless, conserved motifs for two-component phosphor-relay systems have been identified in ethylene receptors, phytochrome photoreceptors, and in a putative osmosensor (Pischke, 2006). The importance of multistep two-component phosphorelay has been investigated in *Escherichia coli*, yeasts and plants through transient expressions as well as in the leaf protoplast system. These studies have provided compelling evidence for a cytokinin role as a major sensing and propagating signal from a wide variety of external and/or internal stimuli such as ethylene, cytokinin, and osmolarity (Pischke, 2006).

Ethylene signaling pathway in *Citrus*

In *Arabidopsis*, there are five members of the ETR family, denoted as ETR1, ETR2, EIN4, ERS1 and ERS2, classified into two subfamilies: subfamily I (ETR1 and

ERS1) and subfamily II (ETR2, EIN4 and ERS2) (Klee, 2004). In tomato, the predicted structures of the described ethylene receptors (LeETR1, LeETR2, NR, LeETR4, LeETR5 and LeETR6) are very similar to those in *Arabidopsis* (Klee, 2004). Within the CitEST database, we found several members of the ETR family (Table 3). A total

of 63 unique reads related to ETR-like receptors were clustered into 5 contigs and 2 singlets. The 5 contigs encode 3 receptors similar to subfamily I members (two ETR1 and one ERS1) and 2 receptors similar to subfamily II members (one EIN4 and one ETR5). These ETR-like contigs were representatively assembled from 7 up to 20

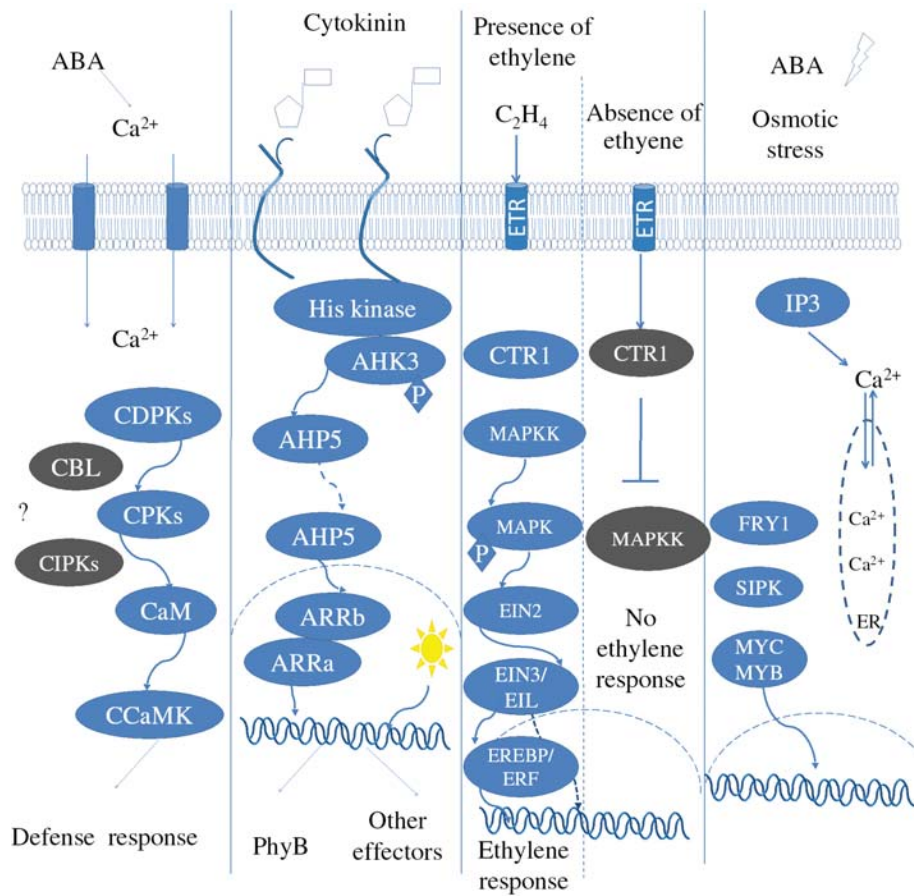


Figure 1 - Schematic representation of signaling cascades in Citrus. A. Calcium signaling components: CDPK (calcium-dependent protein kinase), CPK (calcium protein kinase), CaM (calmodulin), CCaMK (calcium- and calmodulin-dependent protein kinase), CBL (calcium sensor calcineurin B-like protein), CIPKs (CBL interacting protein kinases); B. cytokinin components: AHK3 (histidine kinase), AHP5 (*Arabidopsis* histidine phosphotransfer), ARRa and ARRb (*Arabidopsis* response regulator); C. Ethylene components: ETR (Ethylene receptor), CTR (Constitutive triple response), MAPKK (mitogen-activated protein kinase kinases), MAPK (mitogen-activated protein kinase), EIN (Ethylene insensitive), ERF (Ethylene response factor); D. Abscisic acid: IP3 (inositol triphosphate), FRY1(*fiery1*), SPK (sphingosine kinase), ER (Endoplasmic Reticulum).

Table 2 - Ortholog genes of Cytokinin signaling pathways found in the Citrus EST database.

	Gene	Gene product	Organism	Accession #	Clusters	
					Contigs	Singlets
Cytokinin	<i>AHK3</i>	AHK3	<i>Arabidopsis thaliana</i>	At1g27320	2	2
	<i>AHP5</i>	AHP3	<i>Arabidopsis thaliana</i>	At5g39340	1	1
	<i>ARR - A type</i>	ARR6	<i>Zea mays</i>	BAB20581.1	1	1
		ARR3	<i>Dianthus caryophyllus</i>	AAK14395.1	1	0
		ARR11	<i>Oryza sativa</i>	BAD82798.1	1	0
	<i>ARR - B type</i>	ARR1	<i>Arabidopsis thaliana</i>	BAA74528.1	1	0
		ARR2	<i>Arabidopsis thaliana</i>	gi11357178	2	1

reads, originated from different cDNA libraries mostly from fruits, but also from seeds, infected and non-infected leaves and bark. It seems the ETR-like contigs mostly represent expression within healthy plant tissues (especially fruits) and only in a few cases they originated from *Xylella fastidiosa*, *Citrus tristeza virus* (CTV) or *Phytophthora spp.* infected tissues. This is consistent with the fact that the ethylene transduction pathway is activated both during fruit ripening and biotic stresses. Moreover, the majority of the ETR-like contigs were from *Citrus sinensis* origin, and secondarily from either *Citrus reticulata* or *Poncirus trifoliata*. None of the citrus ETR-like contigs presented a complete sequence when aligned with their best hit in Blast searches. Interestingly, the sequence of the contig encoding for a citrus ERS1 was 99% identical to a sequence of *C. sinensis* previously described. No ETR2-like or ERS2-like receptors were observed within the CitEST database. Further studies are necessary to better characterize the complete family of ethylene receptors of citrus species and their expression pattern during plant development and under stress conditions.

Analyses of the CitEST database revealed 6 unique reads related to CTR1, clusterized into 1 contig and 4 singlets (Table 3). Interestingly, these 4 singlets were all originated from *C. reticulata* cDNA libraries: 1 singlet from a fruit library and 3 singlets from a *Xylella fastidiosa*-infected leaf library. The only citrus CTR1-like contig, originated from *Citrus aurantifolia* leaves, encodes an incomplete ORF (Table 3).

There were 22 unique reads related to EIN2 within the CitEST database, clusterized into 3 contigs and 1 singlet (Table 3). The citrus EIN2-like contigs are formed from various cDNA libraries, mostly from *C. sinensis*, but also from *C. reticulata*, *C. aurantium* and *P. trifoliata*. These contigs represent EIN2 sequences expressed in fruits, *Phytophthora sp.*-infected bark, healthy leaves, *Xylella fastidiosa*- or CTV-infected leaves, which are most homologous to EIN2 proteins from *Petunia x hybrida* and *Lycopersicon esculentum*.

In *Arabidopsis*, there are 6 members of the EIN3 family, where EIN3 and EIL1 are the most thoroughly related ones (Alonso *et al.*, 2003). We found 55 EIN3-like unique reads within the CitEST database clusterized into 4 contigs and 6 singlets (Table 3). These 6 singlets encode incomplete ORFs from *C. sinensis*, *C. reticulata* and *C. latifolia*, either leaves or fruits reads. Most of the contigs originated from *C. sinensis* and *C. reticulata*, infrequently from *P. trifoliata*, and notably, also from leaves infected with *Xylella fastidiosa* and fruits of healthy plants.

Among the main ethylene signaling pathway genes, the EREBP-like transcription factors were the most abundant within the CitEST database. A total of 159 unique reads related to EREBP-like proteins were found within the CitEST database and clusterized into 20 contigs and 14 singlets (Table 3). There were 6 singlets encoding incom-

plete ERF-like ORFs from *C. sinensis*, *C. reticulata*, *C. latifolia* and *P. trifoliata*, from fruits, seeds, but mostly from either healthy or *X. fastidiosa*-infected leaves. Among the 11 citrus ERF-like contigs, only 4 ERF-like contigs seem complete. The 8 EREBP-like singlets found within CitEST encode incomplete ORFs from *C. sinensis* or *C. reticulata*, from either fruits or leaves. Searches within the CitEST revealed 9 citrus EREBP-like contigs, among which only the contigs homologous to the *Arabidopsis* EREBP-like factors (gb|AAM64362, emb|CAB96654 and ref|NP_197901) seem complete. The ERF-like and EREBP-like contigs originated from a large variety of citrus species and plant organs, either under no stress or under biotic stress situations.

In conclusion, the CitEST database is fairly representative for ethylene signaling pathway genes. Despite the fact that most of the sequences found within the CitEST database do not correspond to a complete ORF, there are several members related to each of the pathway steps (Figure 1C). In general, the ethylene signaling genes present in the CitEST database represent expression that is consistent with the many physiological processes and responses associated with this transduction pathway within both healthy plant tissues, as well as in tissues under biotic stress. Further investment is necessary to clone complete citrus ethylene signaling sequences, validate them and better characterize their expression patterns during plant development and under stress conditions.

ABA signaling pathway in *Citrus*

The role of sugar and phytohormones such as ABA and ethylene has been investigated in citrus, and several important functions have been attributed to these compounds. Crosstalk between sugar, ABA and ethylene pathways has been proposed; however, only a few genes involved in the signaling have been previously reported. The signal transduction that leads to the multiple responses regulated by ABA has been revealed through genetic and physiological analyses in the plant model *Arabidopsis thaliana*. In these studies, several different genes have been associated with the ABA response including *era1*, which encodes the beta-subunit of protein farnesyltransferase (PFT) and PGGT-I (Johnson *et al.*, 2005), the gene encoding the beta-subunit of protein geranylgeranyltransferase type I (PGGT I). These genes are involved in protein prenylation and their products may be partially redundant in the ABA response (Johnson *et al.*, 2005). Inositol 1,4,5-triphosphate dephosphorylation is also important in this signal transduction since mutation in *fry1*, the inositol polyphosphate 1-phosphatase encoding gene, leads to super-induction of ABA- and stress-responsive genes (Xiong *et al.*, 2002). Other genes involved in the ABA response participate in the RNA metabolism like *hyl1*, a gene encoding a double-stranded RNA binding protein that is related to activity of MAP kinases (Lu *et al.*, 2002); *abh1* (Hugouvieux *et al.*, 2001), a

Table 3 - Ortholog genes of Ethylene signaling pathways found in the Citrus EST database.

Gene	Gene product	Organism	Accession #	Clusters	
				Contigs	Singlets
ETR-like ¹	ETR1	<i>Mangifera indica</i>	gb AAF61919	2	0
		<i>Pyrus communis</i>	gb AAL66207	0	1
	ETR5	<i>Lycopersicon esculentum</i>	gb AAU34077	1	0
	ERS1	<i>Citrus sinensis</i>	gb AAC99435	1	1
	EIN4	<i>Fragaria x ananassa</i>	emb CAC48386	1	0
CTR1 ²	CTR1	<i>Pyrus communis</i>	gb AAL66190	1	0
		<i>Rosa hybrid cultivar</i>	gb AAK40361	0	4
EIN2 ³	EIN2	<i>Petunia x hybrida</i>	gb AAR08678	2	0
		<i>Lycopersicon esculentum</i>	gb AAS67011	1	0
EIN3-like	EIN3	<i>Arabidopsis thaliana</i>	gb NP_195948	0	1
		<i>Fagus sylvatica</i>	emb CAC09582	1	0
		<i>Cucumis melo</i>	dbj BAB64344	1	0
		<i>Cucumis melo</i>	dbj BAB64345	2	0
		<i>Cucumis melo</i>	dbj BAB64345	0	3
Ethylene	EIL ⁴	<i>Nicotiana tabacum</i>	gb AAP03997	0	1
		<i>Nicotiana tabacum</i>	gb AAP04000	0	1
		<i>Lycopersicon esculentum</i>	gb AAK58858	0	1
		<i>Fagus sylvatica</i>	emb CAE54591	3	0
	ERF ⁶	<i>Cucumis melo</i>	dbj BAD01556	1	0
		<i>Lycopersicon esculentum</i>	gb AAS72389	1	0
		<i>Nicotiana sylvestris</i>	sp Q9LW49	1	0
		<i>Nicotiana sp.</i>	sp Q9SXS8	1	0
		<i>Gossypium hirsutum</i>	gb AAV51937	2	2
		<i>Gossypium hirsutum</i>	gb AAV51938	1	0
		<i>Gossypium hirsutum</i>	gb AAO59439	1	0
		<i>Gossypium barbadense</i>	gb AAT77191	0	1
EREBP-like ⁵	<i>Arabidopsis thaliana</i>	ref NP_182011	0	1	
	<i>Nicotiana tabacum</i>	sp Q9SXS8	0	1	
	<i>Vitis aestivalis</i>	gb AAQ96342	0	1	
	<i>Lycopersicon esculentum</i>	gb AAC49740	1	0	
	<i>Nicotiana tabacum</i>	pir T03927	1	0	
	<i>Nicotiana tabacum</i>	dbj BAA07324	1	0	
	<i>Arabidopsis thaliana</i>	gb AAM64362	1	0	
	<i>Arabidopsis thaliana</i>	dbj BAA97157	1	0	
	<i>Arabidopsis thaliana</i>	emb CAB96654	1	0	
	<i>Arabidopsis thaliana</i>	ref NP_197901	1	0	
	<i>Arabidopsis thaliana</i>	gb AAO00938	1	0	
	<i>Arabidopsis thaliana</i>	gb AAM65925	1	0	
EREBP	<i>Arabidopsis thaliana</i>	gb AAM14242	0	1	
	<i>Arabidopsis thaliana</i>	gb AAP06820	0	1	
	<i>Arabidopsis thaliana</i>	ref NP_568755	0	1	
	<i>Arabidopsis thaliana</i>	ref NP_177022	0	1	
	<i>Arabidopsis thaliana</i>	ref NP_196348	0	1	
	<i>Arabidopsis thaliana</i>	ref NP_196895	0	1	
	<i>Oryza sativa</i>	emb CAE05154.2	0	1	
	<i>Mesembryanthemum crystallinum</i>	gb AAP80810	0	1	

¹Ethylene receptor. ²Constitutive triple response. ³Ethylene insensitive. ⁴Ethylene insensitive 3-like. ⁵Ethylene response element binding protein-like.⁶Ethylene response factor.

gene encoding an mRNA cap binding protein that modulates the ABA signaling by affecting transcription of early ABA signaling elements; and *sad1*, that encodes a polypeptide similar to multifunctional Sm-like snRNP proteins required for mRNA splicing, export and degradation (Xiong *et al.*, 2002). A protein that works as a receptor for ABA, encoded by the *fca* gene has recently been identified and is related to the control of flowering time in *Arabidopsis thaliana* (Razem *et al.*, 2006). Several genes were found in

the Citrus EST database (Table 4) and some are shown in Figure 1D.

The involvement of ethylene and ABA in citrus leaf abscission has also been reported. It has been proposed that leaf abscission enhancement induced by ABA can result from the direct effect of ABA on ethylene biosynthesis. This process occurs when citrus plants are rewatered after a period of water stress. ABA, the primary sensitive signal to water stress, accumulates in the roots and modulates the synthesis of root 1-aminocyclopropane-1-carboxylic acid

Table 4 - Ortholog genes of Abscisic acid signaling pathways found in the Citrus EST database.

Path	Gene	Gene product	Organism	Accession #	Clusters	
					Contigs	Singlets
Abscisic acid	rbohD/F	NADPH oxidase	<i>Nicotiana tabacum</i>	gi 20805911	1	4
	abi8/kob1	SDL-1	<i>Nicotiana glauca</i>	emb CAD21166.1	3	3
	abh1	CBP80	<i>Arabidopsis thaliana</i>	gb AAF76167.1	2	1
	sad1	SM protein	<i>Arabidopsis thaliana</i>	gb AAM65292.1	2	1
	gpa1	G protein	<i>Lotus corniculatus</i>	emb CAA54467.1	1	1
	gpa2	G protein	<i>Arabidopsis thaliana</i>	gb AAC23761.2	1	0
			<i>Arabidopsis thaliana</i>	ref NP_174475.1	1	0
			<i>Arabidopsis thaliana</i>	gi 3201682	0	1
	rcn1	PP2A	<i>Arabidopsis thaliana</i>	gb AAP37715.1	2	0
			<i>Medicago sativa</i>	gi 11094365	0	3
			<i>Oryza sativa</i>	ref XP_450276.1	1	0
	ost1	kinase	<i>Oryza sativa</i>	gb AAO65504.1	3	0
			<i>Fagus sylvatica</i>	emb CAE54075.1	1	0
			<i>Nicotiana tabacum</i>	gb AAL89456.1	1	0
			<i>Arabidopsis thaliana</i>	dbj BAB08630.1	1	0
			<i>Oryza sativa</i>	gb AAP55046.1	1	0
			<i>Arabidopsis thaliana</i>	emb CAC87047.1	1	0
	era1	farnesyltransferase beta subunit	<i>Catharanthus roseus</i>	gb AAQ02809.1	1	0
			<i>Arabidopsis thaliana</i>	dbj BAA96901.1	2	1
	fry1	3'(2'),5'-biphosphate nucleotidase	<i>Arabidopsis thaliana</i>	dbj BAB01188.1	2	1
			<i>Oryza sativa</i>	ref XP_482139.1	1	0
	hyl1	double-stranded RNA-binding protein	<i>Arabidopsis thaliana</i>	gi 22331912	0	1
			<i>Arabidopsis thaliana</i>	gi 22135900	1	0
	gca2	SF16 protein	<i>Arabidopsis thaliana</i>	gb AAO64059.1	1	0
			<i>Arabidopsis thaliana</i>	gb AAN18171.1	1	0
			<i>Oryza sativa</i>	gb AAV33309.1	1	0
<i>Arabidopsis thaliana</i>			gi 11994738	0	1	
ggb	geranylgeranyltransferase I	<i>Glycine max</i>	gb AAQ62584.1	1	0	
		<i>Arabidopsis thaliana</i>	gb AAP37823.1	1	0	
ger1	G-protein-coupled receptor	<i>Arabidopsis thaliana</i>	gb AAD49769.1	1	0	
		<i>Arabidopsis thaliana</i>	gi 15221138	0	1	
fca	Flowering time control protein	<i>Arabidopsis thaliana</i>	emb CAB78672.1	1	0	
		<i>Arabidopsis thaliana</i>	gi 30690648	0	1	
		<i>Arabidopsis thaliana</i>	gi 2204089	0	1	

(ACC) and the increase in ethylene that triggers the events leading to leaf abscission (Katz *et al.*, 2004).

Sugar signaling pathways in *Citrus*

In the Citrus EST database, several genes involved in sugar sensing and signaling were found, including HXK, FRK, SnRK and SNF4 (Table 5). All organisms need to adapt to sugar availability, which is achieved by the ability to respond to sugar levels or flux. Sugar signaling pathways are part of cellular regulatory networks and do not function in isolation. Recent studies have shown evidence of interactions between sugar and phytohormone response; however, little is known about the mechanisms by which different response pathways interact. The function of each sugar signaling gene identified in CitEST must be further investigated in experimental studies in order to associate them to specific biological conditions.

Jasmonic acid signaling pathway in *Citrus*

Jasmonate mediates many transcriptional responses in plants related to wounding and pathogenesis by acting as potent regulators for the expression of numerous frontline

immune response genes, including those for defensins and antifungal proteins. Two multiprotein complexes, COP9 signalosome (CNS) and the SCF (COI1), both play a central role in jasmonate signaling. The JA pathway has been identified through mutant screening. It has been suggested that JA signaling starts with exogenous and endogenous elicitors that lead to JA synthesis. Once JA sensors are activated an intricate network is switched on and as a result JA signaling targets cell response with defense mechanisms through PR protein transcription (Liechti and Farmer, 2006).

In the citrus EST database, some of the genes involved in JA signaling were found (Table 6). Most ESTs were expressed in *X. fastidiosa*-infected or fruit development libraries. Only one read showing similarity to COI1 was encountered in the *X. fastidiosa*-infected library and the citrus sequence showed 78% identity to the COI1 of *A. thaliana*. These results provide another key to understanding the fine control of gene expression in immune responses, and indicate that JA might have a fundamental role in the initiation and maintenance of long-distance signal

Table 5 - Ortholog genes of sugar signaling pathways found in the Citrus EST database.

Gene	Gene product	Organism	Accession #	Clusters	
				Contigs	Singlets
HXK	Hexokinase-1	<i>Arabidopsis thaliana</i>	Q42525	2	0
	Hexokinase-2	<i>Arabidopsis thaliana</i>	P93834	1	0
	hexokinase-related protein 1	<i>Solanum tuberosum</i>	gi 18026821	0	2
	hexokinase -related	<i>Arabidopsis thaliana</i>	gi 15222973	0	1
Sugars	SCRK	<i>Solanum tuberosum</i>	P37829	6	0
	Pyrophosphate-fructose 6-phosphate 1-phosphotransferase alpha subunit	<i>Ricinus communis</i>	Q41140	2	0
	Pyrophosphate-fructose 6-phosphate 1-phosphotransferase alpha subunit	<i>Ricinus communis</i>	Q41141	1	1
	pyrophosphate-dependent phosphofructo-1-kinase	<i>Arabidopsis thaliana</i>	AT4g26270	1	0
	Pyrophosphate-dependent phosphofructo-1-kinase	<i>Arabidopsis thaliana</i>	Q9STQ7	1	0
	Putative pyrophosphate-dependent phosphofructo-1-kinase	<i>Arabidopsis thaliana</i>	gb AAK64113.1	1	0
	pyrophosphate-dependent phosphofructokinase alpha subunit	<i>Citrus x paradisi</i>	gi 3790102	0	2
	fructokinase	<i>Lycopersicon esculentum</i>	gi 23476263	0	1
	fructokinase-like protein	<i>Cicer arietinum</i>	gi 20975618	0	1
	pfkB type carbohydrate kinase protein family	<i>Arabidopsis thaliana</i>	gi 30688079	0	2
SnRK	SNF1-related protein kinase catalytic alpha subunit	<i>Arabidopsis thaliana</i>	Q38997	2	0
	CBL-interacting protein kinase 14 (CIPK14)	<i>Arabidopsis thaliana</i>	gi 15241067	0	1
	CBL-interacting protein kinase 6 (CIPK6)	<i>Arabidopsis thaliana</i>	gi 15235768	0	1
	CBL-interacting protein kinase 23	<i>Arabidopsis thaliana</i>	gi 18397430	0	1
SNF4	putative activator subunit of SNF1-related protein kinase SNF4	<i>Arabidopsis thaliana</i>	AAG10141	2	0
	SNF4b	<i>Medicago truncatula</i>	gi 32364484	0	1

transfer in response to wounding, regulation of fertility, among other processes.

COP9 in *Citrus*

The CSN complex participates in multifaceted cellular processes including regulation of plant development and ubiquitin-mediated proteolysis. Furthermore, the COP9 signalosome shares homologies with the lid sub-complex of the proteasome and is evolutionarily conserved from fission yeast to humans. In the citrus EST database, we investigated the presence of ortholog genes and COP9

similar sequences. Consistent with this possibility, several contigs and singlets among 70 reads in the databank were found (Table 7). All components of COP9 multicomplex were identified but only the sequence encoding to CSN4 gene was full-length. All reads were equally distributed among the cDNA libraries. Our findings suggest that in *Citrus* the COP9 might have an important role in development and other cellular functions.

The COP9 signalosome (CSN) has been implicated in two distinct processes: regulation of protein degradation through deneddylation of the cullin subunit of multiple

Table 6 - Ortholog genes of Jasmonic Acid signaling pathways found in the Citrus EST database.

	Gene	Gene product	Organism	Accession #	Clusters	
					Contigs	Singlets
Jasmonic acid	<i>COL1</i>	Coronatine insensitive 1	<i>Arabidopsis thaliana</i>	gi 18405209	0	1
	<i>SKP1</i>	Skp1-related protein	<i>Arabidopsis thaliana</i>	At1g75950	1	0
		Skp1	<i>Capsicum annuum</i>	gi 62467589	1	0
		Skp1	<i>Medicago sativa</i>	gi 4959710	1	0
		SKP1	<i>Nicotiana tabacum</i>	gi 51292007	1	0
		Skp1/Ask1-like protein	<i>Zantedeschia</i> hybrid cultivar	gi 47176688	1	0
		SKP1	<i>Brassica napus</i>	gi 81248477	1	0
		SKP1 family protein	<i>Arabidopsis thaliana</i>	gi 18411999	0	1
	<i>CUL1</i>	Cullin-1	<i>Arabidopsis thaliana</i>	Q94AH6	2	0
		putative cullin	<i>Arabidopsis thaliana</i>	gb AAM14063.1	1	0
		putative cullin3	<i>Oryza sativa</i>	XP_467770.1	2	0
		putative cullin protein	<i>Olea europaea</i>	gb AAL27655.2	1	0
		cullin, putative	<i>Arabidopsis thaliana</i>	NP_177125.1	1	0
		Putative cullin	<i>Oryza sativa</i>	gi 14091839	0	1
		CUL1	<i>Oryza sativa</i>	gi 54290813	0	1
		cullin-like protein1	<i>Pisum sativum</i>	gi 22335691	0	2
		cullin 1 protein -related	<i>Arabidopsis thaliana</i>	gi 18411983	0	1
		putative cullin	<i>Arabidopsis thaliana</i>	gi 20268719	0	1
		cullin-like protein	<i>Oryza sativa</i>	gi 34914728	0	1
		<i>RBX1A</i>	RING-box protein 1a (RBX1a-At)	<i>Arabidopsis thaliana</i>	Q940X7	1
		ring-box protein - like	<i>Arabidopsis thaliana</i>	gi 18420256	0	1

Table 7 - Ortholog genes of COP9 signalosome pathways found in the Citrus EST database.

	Gene	Gene product	Organism	Accession #	Clusters	
					Contigs	Singlets
COP9 signalosome	<i>CSN1</i>	FUS1	<i>Arabidopsis thaliana</i>	gb AAK93733.1	1	0
	<i>CSN2</i>	Csn2	<i>Oryza sativa</i>	dbj BAD81083.1	1	1
	<i>CSN3</i>	Csn3	<i>Nicotiana benthamiana</i> <i>Oryza sativa</i>	gb AAO85512.1 ref XP_479811.1	2	0
	<i>CSN4</i>	Csn4/COP8	<i>Arabidopsis thaliana</i>	gb AAL58103.1	1	0
		Csn5a	<i>Lycopersicon esculentum</i>	gb AAG43411.1	1	0
	<i>CSN5</i>	Csn5b/ Fusca5	<i>Arabidopsis thaliana</i>	AT1G71230	1	1
	<i>CSN6</i>	Csn6	<i>Arabidopsis thaliana</i>	AT5g56280	1	0
	<i>CSN7</i>	Csn7	<i>Arabidopsis thaliana</i>	At1g02090	2	0
<i>CSN8</i>	Cns8/ FUS4	<i>Arabidopsis thaliana</i>	NP_199111.1	1	0	

SCF (Skp1/cullin/F-box) E3-ubiquitin ligases and modulation of kinase signaling pathways through associated kinases. Although the clear mechanism of action of COP9 is not yet clarified, it has been demonstrated in *Arabidopsis* seedling that COP9 genes play a key role in the light control of development, integrating light signals and modulating developmental pattern formation (Chamovitz and Yahaalom, 2003). In this study, the authors have systematically investigated COP/DET/FUS-controlled genome expression during *Arabidopsis* seedling development using a cDNA microarray.

COP9 is an intriguing subject of study and many questions remain in our findings. Further studies would be necessary to clarify the role of COP9 in Citrus.

Kinases and phosphatases in Citrus

Analyses of the *Arabidopsis* genome show 20 genes that might encode MAPKs, 10 genes that appear to encode MAPKKs and more than 60 genes possibly encoding MAPKKK homologs (Champion *et al.*, 2004).

In CitEST, we did find contigs and singlets related to different MAP kinases (Table 8), including the tobacco NPK1 (MAPKKK), NQK1 (MAPKK) and NRK1 (MAPK) which is the cascade involved in the regulation of cytokinesis in plant cells (Soyano *et al.*, 2003), the YDA MAPKK kinase that plays a key role in the early development of *Arabidopsis* embryos (Lukowitz *et al.*, 2004), and the tobacco WIPK (wounding-induced protein kinase)

Table 8 - Ortholog genes of kinase and phosphatase signaling pathways found in the Citrus EST database.

Gene	Gene product	Organism	Accession #	Clusters		
				Contigs	Singlets	
MAPK	AtMPK3	<i>Arabidopsis thaliana</i>	Q39023	1	0	
	ATMPK16	<i>Arabidopsis thaliana</i>	NP_197402	2	1	
	ATMPK18	<i>Arabidopsis thaliana</i>	NP_175756	0	1	
	MAPK	<i>Medicago sativa</i>	AAD28617	2	0	
	MAPK2	<i>Glycine max</i>	AAQ14867	1	0	
	MAPK7	<i>Oryza sativa</i>	BAD61401	1	0	
	MAP kinase	<i>Arabidopsis thaliana</i>	D84859	1	0	
	MAP kinase 4	<i>Petroselinum crispum</i>	AAN65180	2	0	
	MPK8	<i>Arabidopsis thaliana</i>	NP_173253	0	1	
	MPK17	<i>Arabidopsis thaliana</i>	AAP21277	1	0	
	MPK9	<i>Brassica napus</i>	AAU95462	1	0	
	NRK1	<i>Nicotiana tabacum</i>	BAB32406	1	0	
	NTF3	<i>Nicotiana tabacum</i>	CAA49592	2	0	
	NTF6	<i>Nicotiana tabacum</i>	Q40531	0	1	
	RMAPK1	<i>Oryza sativa</i>	AAF23902	0	1	
	WIPK	<i>Nicotiana tabacum</i>	BAB79636	1	0	
	MAPKK	MAPKK	<i>Lycopersicon esculentum</i>	AAU04436	2	0
		NQK1 MAPKK	<i>Nicotiana tabacum</i>	BAB32405	1	0
Non-receptor protein kinase	CTR1	<i>Arabidopsis thaliana</i>	CAB82938	1	0	
	CTR1	<i>Brassica juncea</i>	AAP86285	0	1	
	CTR1	<i>Brassica juncea</i>	AAP86286	3	1	
	CTR1	<i>Oryza sativa</i>	BAC79157	0	1	
	CTR1	<i>Oryza sativa</i>	BAD28881	1	0	
	CTR1	<i>Oryza sativa</i>	BAD37611	4	1	
	CTR1	<i>Oryza sativa</i>	XP_450193	13	0	
	CTR1	<i>Rosa hybrid cultivar</i>	AAK40361	2	3	
	EDR1	<i>Arabidopsis thaliana</i>	AAG31143	0	2	
	EDR1	<i>Oryza sativa</i>	BAD62538	1	0	
	MAP3K	<i>Arabidopsis thaliana</i>	CAB16796	1	0	
	MAP3K	<i>Arabidopsis thaliana</i>	D85436	0	1	
	MAP3K alpha 1	<i>Oryza sativa</i>	BAD27776	1	0	
	MAP3K delta-1	<i>Arabidopsis thaliana</i>	CAA74591	0	1	
	MAP3K delta-1	<i>Arabidopsis thaliana</i>	CAB87658	1	0	
	MAP3K delta-1	<i>Arabidopsis thaliana</i>	NP_196746	0	1	
	MAP3K delta-1	<i>Oryza sativa</i>	XP_464691	1	0	
MAP3K epsilon	<i>Arabidopsis thaliana</i>	BAB01760	2	0		
MAP3K epsilon 1	<i>Brassica napus</i>	CAB54520	0	1		
MAP3K gamma	<i>Arabidopsis thaliana</i>	CAA74696	3	0		
MEK kinase	<i>Arabidopsis thaliana</i>	AAD10848	0	2		
MEKK1	<i>Arabidopsis thaliana</i>	CAB77975	2	0		

Table 8 (cont.)

Gene	Gene product	Organism	Accession #	Clusters		
				Contigs	Singlets	
MAPKKK	mekk1	<i>Medicago sativa</i>	CAE00640	1	0	
	NPK1-related protein kinase 2	<i>Arabidopsis thaliana</i>	BAA21856	1	0	
	YDA	<i>Arabidopsis thaliana</i>	AAR10436	2	0	
	MAP kinase	<i>Arabidopsis thaliana</i>	BAB01779	1	0	
	mitogen-activated protein kinase	<i>Nicotiana tabacum</i>	AAQ83971	1	0	
	similar to MAP/ERK kinase kinase 3 gij4505153	<i>Arabidopsis thaliana</i>	NP_175344	0	2	
	ZIK1	<i>Medicago sativa</i>	CAC84087	13	5	
At2g20040	cAMP-dependent protein kinase - catalytic	<i>Arabidopsis thaliana</i>	AAD24392	1	0	
DWARF12	glycogen synthase kinase 3 beta protein kinase	<i>Arabidopsis thaliana</i>	AAN71719	1	1	
GSK1	GSK3	<i>Arabidopsis thaliana</i>	AAB71545	0	1	
GSK-3	glycogen synthase kinase 3	<i>Medicago sativa</i>	CAC08564	1	1	
GSK-3	GSK-3-like protein MsK4	<i>Medicago sativa</i>	AAN63591	4	0	
MSK-1	Glycogen synthase kinase-3 homolog MsK-1	<i>Medicago sativa</i>	P51137	0	3	
MSK-3	protein kinase MSK-3-like	<i>Arabidopsis thaliana</i>	CAB87631	2	3	
MSK-3	Glycogen synthase kinase-3 homolog MsK-3	<i>Medicago sativa</i>	P51139	3	1	
AT4G28880	casein kinase I	<i>Arabidopsis thaliana</i>	NP_194617	1	0	
At3g13670	putative casein kinase	<i>Arabidopsis thaliana</i>	AAM51279	8	3	
P0510F09.20	putative casein kinase I	<i>Oryza sativa</i>	NM_191682	6	1	
CKA1	casein kinase II alpha chain 1	<i>Arabidopsis thaliana</i>	NP_201539	2	2	
ck2beta2	Casein kinase II regulatory subunit	<i>Nicotiana tabacum</i>	CAD32500	4	5	
	casein kinase II alpha subunit	<i>Zea mays</i>	AAF76187	1	0	
CIPK	CBL-interacting protein kinase	<i>Brassica napus</i>	AAL37170	2	0	
CK1	CIPK-like protein 1	<i>Oryza sativa</i>	Q6X4A2	2	0	
CIPK1	CBL-interacting protein kinase 1	<i>Arabidopsis thaliana</i>	NP_566580	4	1	
CIPK2	CBL-interacting protein kinase 2	<i>Arabidopsis thaliana</i>	AAF86506	1	0	
CIPK3	CBL-interacting protein kinase 3	<i>Arabidopsis thaliana</i>	NP_850093	0	1	
CIPK5	CBL-interacting protein kinase 5	<i>Arabidopsis thaliana</i>	NP_568241	2	0	
CIPK6	CBL-interacting protein kinase 6	<i>Arabidopsis thaliana</i>	NP_194825	1	1	
CIPK8	CBL-interacting protein kinase 6	<i>Arabidopsis thaliana</i>	AAK16683	0	1	
CIPK9	CBL-interacting protein kinase 9	<i>Arabidopsis thaliana</i>	NP_171622	3	2	
CIPK10	CBL-interacting protein kinase 10	<i>Arabidopsis thaliana</i>	NP_568878	0	2	
CIPK11	CBL-interacting protein kinase 11	<i>Arabidopsis thaliana</i>	O22932	1	0	
CIPK14	CBL-interacting protein kinase 14	<i>Arabidopsis thaliana</i>	AAK16689	0	1	
CIPK18	CBL-interacting protein kinase 18	<i>Arabidopsis thaliana</i>	NP_174217	1	0	
CIPK20	CBL-interacting protein kinase 20	<i>Arabidopsis thaliana</i>	NP_199394	1	1	
CIPK22	CBL-interacting protein kinase 22	<i>Arabidopsis thaliana</i>	NP_181383	1	0	
CIPK23	CBL-interacting protein kinase 23	<i>Arabidopsis thaliana</i>	NP_564353	2	2	
CIPK24	CBL-interacting protein kinase 24	<i>Arabidopsis thaliana</i>	AAK72257	2	0	
CIPK25	CBL-interacting protein kinase 25	<i>Arabidopsis thaliana</i>	NP_568466	1	2	
Protein phosphatases	PP1	protein phosphatase PP1 isozyme 2	<i>Arabidopsis thaliana</i>	BAB09762	1	2
	PP1	protein phosphatase PP1	<i>Phaseolus vulgaris</i>	CAA88254	2	0
	PP1A	phosphoprotein phosphatase 1a catalytic chain	<i>Catharanthus roseus</i>	T09995	0	2
	PP1 beta	protein phosphatase 1, catalytic beta subunit	<i>Medicago sativa</i>	CAA05491	4	3
	PP1	protein phosphatase PP1 isozyme 4	<i>Arabidopsis thaliana</i>	AAB87136	1	0
	ZmPP1	protein phosphatase-1	<i>Zea mays</i>	AAA33545	0	1
	PP2Ac	protein phosphatase 2A-4 catalytic subunit	<i>Arabidopsis thaliana</i>	AAD10855	3	3
	PP2Ac2	protein phosphatase 2A catalytic subunit	<i>Lycopersicon esculentum</i>	AAQ67226	3	0
	PsPP2A	serine/threonine protein phosphatase 2A	<i>Pisum sativum</i>	AAM21172	1	0
	PPX	protein phosphatase 4 catalytic	<i>Malus x domestica</i>	CAA87385	1	0
	LePP5	PP5	<i>Lycopersicon esculentum</i>	AAN64317	1	1
	PP7	PP7	<i>Arabidopsis thaliana</i>	NP_851258	3	0
	PP2A	protein phosphatase 2A 65 kDa regulatory subunit	<i>Arabidopsis thaliana</i>	AAO00848	4	7
	PP2A	phosphatase 2A regulatory A subunit	<i>Oryza sativa</i>	XP_450276	1	1
	PP2C	catalytic/ protein phosphatase type 2C	<i>Arabidopsis thaliana</i>	NP_195118	37	37
	PP2C	protein phosphatase 2C-like	<i>Oryza sativa</i>	BAD72331	9	0
	At5g19280	putative kinase associated protein phosphatase	<i>Arabidopsis thaliana</i>	AAM51227	2	0
	AT1G05000	tyrosine specific protein phosphatase family protein	<i>Arabidopsis thaliana</i>	NP_171993	2	0
	AT4G18593	dual specificity protein phosphatase	<i>Arabidopsis thaliana</i>	NP_567561	0	4

which is involved in the cascade to disease resistance (Yang *et al.*, 2001). Only one contig is related to WIPK and shows a tendency of expression in infected leaf libraries with *X. fastidiosa* and *Citrus tristeza virus* of *C. sinensis* and *P. trifoliata*, respectively, which is consistent with its function in disease resistance (Yang *et al.*, 2001).

In the *Arabidopsis* genome, 112 phosphatase catalytic subunit sequences have been identified, 69 of which are PP2Cs (Kerk *et al.*, 2002). In the CitEST database, 36 contigs and singlets related to PPP family were found covering its major members with the exception of PP2B, which has not been detected in plants until now; and 83 contigs similar to PP2C were found (Table 8).

In CitEST, contigs and singlets related to GSK3 especially to alfalfa GSK3 were found. We also found 16

contigs similar to Ck1 and 7 similar to Ck2, and 4 singlets similar to Ck1 and 7 similar to Ck2. We did find 2 contigs related to Kinase-associated protein phosphatase in CitEST (Table 8). We also found 2 contigs and 1 singlet related to tyrosine-specific protein phosphatase protein and 1 contig and 5 singlets similar to another tyrosine phosphatase called dual-specificity protein phosphatase, which has also been implicated in the negative regulation of MAPK in *Arabidopsis* (Gupta *et al.*, 2002).

Inositol phosphate in *Citrus*

The search in the CitEST database for enzymes involved in inositol metabolism identified components of several steps in this pathway (Table 9, Figure 2). Several contigs related to these enzymes showed high expression in leaves

Table 9 - Ortholog genes of Inositol phosphate pathways found in the Citrus EST database.

Gene	Gene product	Organism	Accession #	Clusters	
				Contigs	Singlets
PI3K	phosphatidylinositol 3-kinase	<i>Brassica napus</i>	AAN62481	3	1
pi3k	phosphatidylinositol 3-kinase	<i>Medicago truncatula</i>	CAD56881	1	1
PI4K	Phosphatidylinositol 4-kinase	<i>Arabidopsis thaliana</i>	CAB37928	3	1
At1g60890	phosphatidylinositol-4-phosphate 5-kinase	<i>Arabidopsis thaliana</i>	AAM91758	4	4
PIPK1	phosphatidylinositol-4-phosphate 5-kinase	<i>Nicotiana rustica</i>	AAF80332	1	0
PIP5K	phosphatidylinositol-4-phosphate 5-kinase	<i>Oryza sativa</i>	AAP55050	4	0
IP5P1	inositol-1,4,5-trisphosphate 5-phosphatase	<i>Arabidopsis thaliana</i>	Q84MA2	5	3
IMPase	inositol-1(or 4)-monophosphatase	<i>Arabidopsis thaliana</i>	NP_186936	2	0
IMP3	inositol-1(or 4)-monophosphatase 3	<i>Lycopersicon esculentum</i>	P54928	1	0
Phospholipase C	Phospholipase C	<i>Arabidopsis thaliana</i>	BAC22506	5	4
		<i>Glycine max</i>	AAB03258	2	1
		<i>Nicotiana rustica</i>	CAA65127	2	2
		<i>Oryza sativa</i>	CAC81703	0	2
plc1					
IMT1	myo-inositol O-methyltransferase	<i>Mesembryanthemum crystallinum</i> (common iceplant)	S22696	1	2
AT4g17370	inositol 2-dehydrogenase	<i>Arabidopsis thaliana</i>	CAB78740	1	0
INPS1	inositol-1-phosphate synthase	<i>Nicotiana paniculata</i>	Q9SSV4	5	0
Inos-1-P_synth	inositol-1-phosphate synthase	<i>Citrus x paradisi</i>	P42802	1	1
OSJNBa0094J08.7	putative multiple inositol polyphosphate phosphatase	<i>Oryza sativa</i>	XP_470115	1	1
P0456F09.5	putative myo-inositol oxygenase	<i>Oryza sativa</i>	BAD53821	1	0
SAL1	Inositol-1,4-bisphosphate 1-phosphatase 1	<i>Arabidopsis thaliana</i>	Q42546	2	2
OJ1548_F12.22	Inositol-1,4-bisphosphate 1-phosphatase 1	<i>Oryza sativa</i>	XP_468288	0	3
At1g03930	putative protein kinase ADK1	<i>Arabidopsis thaliana</i>	AAM20169	2	1
B1114B07.27-1	putative protein kinase ADK1	<i>Oryza sativa</i>	BAD45137	6	1
OSJNBa0032G08.19	Putative phosphoinositide phosphatase	<i>Oryza sativa</i>	XP_470554	3	0
ATG5	phosphoinositide 5-phosphatase	<i>Arabidopsis thaliana</i>	NP_190751	0	2
PTEN	putative Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (PTEN)	<i>Arabidopsis thaliana</i>	AAO13749	3	1

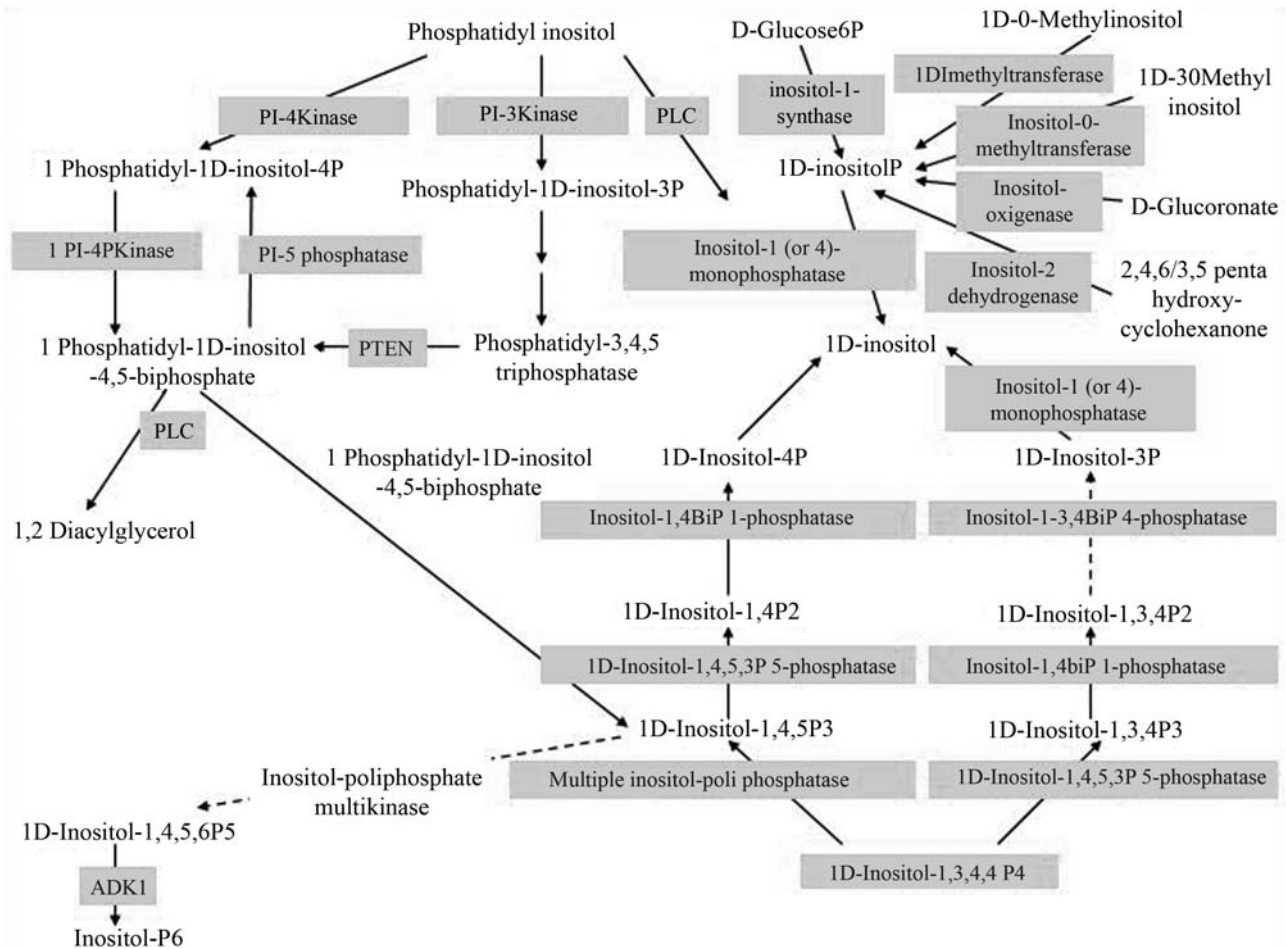


Figure 2 - Putative inositol metabolism in citrus. The enzymes for which contigs and/or singlets have been found in citrus are highlighted in gray.

and fruit libraries. Inositol has been implicated in the early signaling events of plants linking gravity sensing to the initiation of the gravitropic response. However, at present, the contribution of the phosphoinositide signaling pathway in plant gravitropism is not well understood. Recently, Liu *et al.* (2006) reported the role of inositol 1,4,5-trisphosphate IP(3) in transducing heat-shock (HS) signals in *Arabidopsis*. The authors provided the primary evidence for the possible involvement of IP(3) in HS signal transduction in higher plants. Our results suggest that the activity of the inositol pathway might reveal an important role in the cell signaling network.

In our investigation, we found one putative contig with sequence similarity to the catalytic subunit of the cyclic adenosine monophosphate (cAMP) dependent protein kinase and one singlet similar to the regulatory subunit. On the other hand, the cyclic guanidine monophosphate (cGMP) dependent protein kinase was not found in the Citrus EST database.

Plant peptides in Citrus

Although we did not find any ESTs similar to systemin, phytosulfokine and clavata3, we did find a large

number of contigs and singlets for their receptors, which suggests the possible existence of these signaling peptides.

Concluding Remarks

In the present work, we report the presence of several genes involved in the signaling pathways of calcium, sugar and plant hormones in the citrus genome. These results may indicate that similar mechanisms described in other plants, such as *Arabidopsis*, occur in citrus. Further experimental studies must be conducted in order to understand the different signaling pathways present.

An interesting result obtained was that a high number of genes involved in the signaling pathway of ethylene were found in the CitEST database. Despite the fact that most of the sequences found within the CitEST database do not correspond to a complete ORF, there are several members related to each of the pathway steps. In general, the ethylene signaling genes present in the CitEST database represent expression within both healthy plant tissues as well as tissues under biotic stress, coherently with the several physiological processes and responses associated with this transduction pathway.

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Internet Resources

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

Genbank, <http://www.ncbi.nlm.nih.gov/> (November 6, 2006).

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