In silico analysis of phytohormone metabolism and communication pathways in citrus transcriptome

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Abstract

Plant hormones play a crucial role in integrating endogenous and exogenous signals and in determining developmental responses to form the plant body throughout its life cycle. In citrus species, several economically important processes are controlled by phytohormones, including seed germination, secondary growth, fruit abscission and ripening. Integrative genomics is a powerful tool for linking newly researched organisms, such as tropical woody species, to functional studies already carried out on established model organisms. Based on gene orthology analyses and expression patterns, we searched the Citrus Genome Sequencing Consortium (CitEST) database for Expressed Sequence Tags (EST) consensus sequences sharing similarity to known components of hormone metabolism and signaling pathways in model species. More than 600 homologs of functionally characterized hormone metabolism and signal transduction members from model species were identified in citrus, allowing us to propose a framework for phytohormone signaling mechanisms in citrus. A number of components from hormone-related metabolic pathways were absent in citrus, suggesting the presence of distinct metabolic pathways. Our results demonstrated the power of comparative genomics between model systems and economically important crop species to elucidate several aspects of plant physiology and metabolism.

Key words: defense responses, development, plant growth regulators.

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Introduction

Plant growth and development are controlled by the integration of several endogenous and environmental signals. Plant hormones play a crucial role in integrating endogenous and exogenous signals and in determining the final developmental responses to form the plant body. Hormones are molecules that are produced by one specific organ and conveyed to target tissues, where they elicit a physiological response at low concentration (Davies, 1995). This definition does not hold true for most of the plant hormones, which are synthesized by several different tissues or cell types, but can act locally, as well as at a distance. Moreover, the majority of the plant hormones are small, relatively simple molecules (Gray, 2004). Traditionally, plant hormones have been considered to be small lipophilic compounds, such as abscisic acid (ABA), auxin (IAA), brassinosteroids (BR), cytokinins (CK), ethylene (ET), gibberellin (GA), jasmonates (JA) and salicylic acid (SA) (Davies, 1995). Recent evidence from genetic and biochemical studies show the involvement of many secretory and non-secretory peptide signals in many aspects of plant growth regulation, including defense responses, callus growth, meristem organization, self-incompatibility, root growth, leaf-shape regulation, nodule development,
and organ abscission, and comprise a newly-found class of peptide hormones in plants (Matsubayashi and Sakagami, 2006).

Virtually every aspect of plant growth and development is under hormonal control to some extent. A diverse array of cellular and developmental processes may be controlled by single or multiple hormones that may function in concert to control a single process. The application of exogenous hormone has been largely employed to study the function of its endogenous counterparts in plants. This leads to the attribution of general roles for each phytohormone such as the well-characterized function of ethylene in fruit ripening, the regulation of the cell cycle by auxin and CK, the induction of seed germination and stem elongation by GA, and the maintenance of seed dormancy by ABA. Forward genetic approaches have allowed the isolation of hormone biosynthetic and response mutants (Gazzarrini and McCourt, 2003). More recently, biochemical and in silico genome studies have demonstrated the hormonal role of previously unsuspected molecules (Matsubayashi and Sakagami, 2006). An increasing number of genomic tools are being used to probe hormone biosynthesis, transport and response; this integrated approach has contributed to a clearer picture of the mechanisms involved in plant developmental control by hormones.

Plant hormone biosynthesis is closely associated to primary and secondary metabolism. Auxins are tryptophan conjugates and distinct genetic pathways control their biosynthesis (Cohen et al., 2003). CKs are adenine-related purines (Sakakibara, 2006); GAs are tetracyclic diterpenoids synthesized in a complex pathway involving plastids, endoplasmic reticulum and the cytosol (Fleet and Sun, 2005). ABA is synthesized either from MVA (mevalonic acid) or MEP (methylenylthritol-phosphate) (Nambara and Marion-Poll, 2005), especially in vascular bundles and guard cells (Ko Tai et al., 2004). Ethylene is synthesized from methionine by the intermediate S-adenosyl-L-methionine (AdoMet) and 1-aminocyclopropane-1-carboxylate (ACC) (Chae and Kieber, 2005). BR biosynthesis is highly networked and consists of two parallel routes: an early and a late C-6 oxidation pathway, connected at multiple steps, and also linked to an early C-22 oxidation pathway (Fujioka and Yokota, 2003). JA is an oxylipin, consisting in a group of structurally diverse biologically active compounds, generated by the coordinated action of lipases, lipoxigenases, and a group of cytochrome P450 specialized in the metabolism of hydroperoxy fatty acids (Schilmiller and Howe, 2005). Peptide hormones are produced by the proteolytic processing of the C-terminus of a poly-peptide precursor that may be or may be not synthesized through a secretory pathway as in the case of the RAPID ALKALINIZAITON FACTORS (RALF) and RALF like (RALFL) or systemin and phytohormokine, respectively (Matsubayashi and Sakagami, 2006).

The extensive effects of even dimunute concentrations of plant hormones have led to a tight control of function, not only by biosynthesis rate, but also by a plethora of factors, such as availability of receptors, catabolic rate, conversion into inert forms, translocation, and several interconnected steps of signal transduction. Surprisingly, hormone-mediated signal transduction appears to have evolved common themes for distinct phytohormones in plants, such as protein phosphorylation, G-protein and Calcium/calmodulin-mediated signal transduction, and bacterial two-component signaling system-like and regulated proteolysis (Serino and Deng, 2003; Assmann, 2004; Gray, 2004; Mizuno, 2005).

Ethylene and CK are both perceived by plasma membrane-associated receptors, similar to bacterial two-component regulators that contain an intracellular histidine kinase (HK) domain (Stepanova and Alonso, 2005; Sakakibara, 2006). The kinase activity is activated by ligand binding, resulting in self-phosphorylation and initiating a series of phospho-transfer reactions, which culminate in activation of a response regulator protein that functions as the effector component of the pathway (Guo and Ecker, 2004; Ferreira and Kieber, 2005). Ethylene is perceived by a family of five receptors: ETHYLENE RECEPTOR1 (ETR1) and ETHYLENE RESPONSIVE SENSOR1 (ERS1), containing a consensus HK domain, functioning as negative regulators of the pathway. The Raf-like Mitogen-Activated Protein (MAP) Kinase Kinase Kinase, CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) interacts with the receptors and also functions as a negative regulator. The integral membrane protein, ETHYLENE INSENSITIVE2 (EIN2), and the transcription factors EIN3 and EIN-LIKE1 (EIL1) are positive regulators of ethylene signaling, downstream of CTR1. The binding of ethylene to receptors inactivates them and results in down-regulation of CTR1 activity. In the absence of ethylene, transcription factors EIN3 and EIL1 are targeted for degradation by an SKP1/Cullin/F-box protein (SCF) complex (Chae and Kieber, 2005).

Similarly, BR is perceived by a receptor complex of two leucine-rich-repeat receptor-like kinases (LRR-RLKs) that interact with each other (Vert et al., 2005). Activation of the receptor kinases by BR binding leads to the de-phosphorylation and accumulation of two nuclear proteins due to the inhibition of a negative regulator (Vert et al., 2005). In the absence of BR, the negative regulator phosphorylates the nuclear proteins and targets them for degradation by the ubiquitin-dependent proteasome pathway. In the case of auxin, a large family of transcriptional repressors Aux/IAA dimerizes with members of the AUXIN RESPONSE FACTOR (ARF) family of transcription factors, preventing ARFs from activating auxin-responsive genes, reviewed in Woodward and Bartel (2005). Upon auxin stimuli, the receptor TRANSPORT INHIBITOR RESPONSE1 (TIR1), an SCF ubiquitin E3 ligase F-box protein, ubiquitinates Aux/IAA proteins marking them for degradation by the
26S proteasome, thereby de-repressing the response pathway (Parry and Estelle, 2006). In a similar manner, perception of active GAs leads to degradation of various transcriptional repressor proteins containing the DELLA-domain, via an SCF-E3 ubiquitin ligase-SLEEPY1 (SLY1) complex (McGinnis et al., 2003). Interestingly, DELLA protein levels are also regulated by auxin and ethylene, indicating a function as a general regulator of plant growth mediated by several plant hormones (Fleet and Sun, 2005).

Perception and signal transduction mechanisms of plant hormones primarily involved in stress responses also share common themes, which are also shared with development regulatory hormones. The SCF complex mediates JA signaling; the F box protein COI1 (CORONATINE INSUSITIVE1) is part of an SCF complex that includes ARABIDOPSIS SKP1-LIKE1 (ASK1) or ASK2 and CUL-LIN1 (CUL1). However, the transcriptional regulator(s) affected by hormone interaction with SCF complexes remain unknown (Schilmiller and Howe, 2005). ABA transduction pathways are characterized by a plethora of intracellular messengers, reflecting its function in integrating several stress responses and antagonizing pathways via cross-talk (Himmelbach et al., 2003). Accumulation of ABA is controlled by upstream signaling events, and plays a quantitative role in signal transduction (Verslues and Zhu, 2005). However, ABA accumulation can be controlled by several metabolic processes, such as ABA synthesis, catabolism or conjugation, and within each of these metabolic processes there are several genes that may act as rate-limiting factors. These genes may be subjected to feedback regulation by ABA (Verslues and Zhu, 2005). NCED (9-cis-epoxy-carotenoid dioxygenase) catalyses the cleavage of the C25 carotenoids, 9-cis-neoxanthin or 9-cis-violaxanthin, to the C15 ABA-precursor xanthoxin; a reaction proposed to be catalysed by carotenoid dioxygenase (Himmelbach et al., 2003). 9-cis-epoxy-carotenoids are formed in xanthophyll cycles under UV-B irradiation, and are involved in photoprotection (Zhou et al., 2002). The reaction is catalysed by a carotenoid dioxygenase, which is homologous to the enzyme involved in ABA biosynthesis (Himmelbach et al., 2003). The presence of two NCED genes in Arabidopsis is consistent with the presence of two distinct 9-cis-epoxy-carotenoid dioxygenases (Himmelbach et al., 2003). An in silico study of the Arabidopsis genome revealed that the two NCED genes have the same domain structure, and are present in both the Brassicaceae and Solanaeae families (Himmelbach et al., 2003). The presence of two NCED genes in Arabidopsis is consistent with the presence of two distinct 9-cis-epoxy-carotenoid dioxygenases (Himmelbach et al., 2003). An in silico study of the Arabidopsis genome revealed that the two NCED genes have the same domain structure, and are present in both the Brassicaceae and Solanaeae families (Himmelbach et al., 2003). The presence of two NCED genes in Arabidopsis is consistent with the presence of two distinct 9-cis-epoxy-carotenoid dioxygenases (Himmelbach et al., 2003). An in silico study of the Arabidopsis genome revealed that the two NCED genes have the same domain structure, and are present in both the Brassicaceae and Solanaeae families (Himmelbach et al., 2003).

The putative functionality of the deduced amino acid sequence of citrus transcripts, in comparison to their Arabidopsis and other model species homologs, was as-
sessed by genetic distance and phylogenetic studies. Phylogenetic analyses were performed using distance and parsimony methods with PAUP* 4.0b10, using default parameters. Re-sampling bootstrap trees containing 1,000 random samples were constructed using PSIGNFIT. Modular functional domains were employed for genetic distance studies with genes previously characterized as having divergent regions and conserved blocks.

**In silico gene expression analysis**

*In silico* qualitative gene expression profiling was performed using virtual northern blot (VNB) analyses of the citrus EST database. Frequency of reads of each EST contig for a given library was calculated and normalized to the total number of reads from the investigated library and the total number of reads in all libraries. A correlation matrix between EST contigs and libraries was then generated, and gene expression patterns among ESTs and libraries were obtained by hierarchical clustering based on Spearman Rank correlation matrix using Cluster v.2.11 (Eisen et al., 1998), by substituting clusters by their average expression pattern. Graphic outputs were generated using Tree View v.1.6 software.

**Results and Discussion**

**CitEST database survey**

We have performed extensive BLAST and keyword searches of the citrus transcriptome to identify homologs of the genes involved in hormone biosynthesis and signal transduction in citrus. We have searched for genes related to auxin, cytokinin, ethylene, abscisic acid, gibberellic acid, jasmonic acid and peptide hormone biosynthesis, transport and translocation, catabolism and signal transduction pathways. In CitEST databases, 601 assembled sequences and EST singlets sharing significant sequence identity with functionally characterized proteins were identified and analyzed (Table 1, Figure 1). The functional characterization of citrus sequences is virtually equivalent to *Arabidopsis* in cellular component and molecular function (Figure 1, Figure S1 and Figure S2). However, transcripts with enzyme activity are more abundant in citrus whereas those showing transcription factor activity are more abundant in *Arabidopsis* proteome (Figure 1). The significance of these findings remains to be investigated in further functional studies.

**Table 1** - Number of citrus transcripts identified by tBLASTn searches of CitEST databases showing sequence similarity to hormone metabolism and signaling components in model species.

<table>
<thead>
<tr>
<th>Phytohormone</th>
<th>Biosynthesis, metabolism and transport</th>
<th>Signal transduction</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abscisic acid</td>
<td>5 (100%)</td>
<td>24 - (92.3%) - ABH1, ABH2</td>
<td>29</td>
</tr>
<tr>
<td>Auxin</td>
<td>17 (94.1% - CYP707A)</td>
<td>36 (100%)</td>
<td>53</td>
</tr>
<tr>
<td>brassinosteroids</td>
<td>33 (100%)</td>
<td>63 (96.8% - TTL, TRIP1)</td>
<td>96</td>
</tr>
<tr>
<td>Cytokinin</td>
<td>13 (100%)</td>
<td>31 (96.8% - CPC)</td>
<td>44</td>
</tr>
<tr>
<td>Ethylene</td>
<td>18 (100%)</td>
<td>35 (97.1% - EIN2)</td>
<td>53</td>
</tr>
<tr>
<td>gibberellic acid</td>
<td>18 (100%)</td>
<td>14 (87.5% - PHOR1, miR159)</td>
<td>32</td>
</tr>
<tr>
<td>Jasmonic acid</td>
<td>87 (95.6% - 4EXT family)</td>
<td>112 (100%)</td>
<td>199</td>
</tr>
<tr>
<td>Peptide hormones</td>
<td>0</td>
<td>15 (65.2% - TomSys, SCR/SP11, SCRL, IDA, IDL, PLS, CLV3, CLE)</td>
<td>15</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>33 (100%)</td>
<td>47 (97.9% - ACD6)</td>
<td>80</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td>224</td>
</tr>
</tbody>
</table>

*Percentage of identified sequences in comparison to the number of bait sequences searched.*
Abscisic acid

Although leaf abscission is not primarily induced by ABA, but by ethylene, it has been shown that in ‘Cleopatra’ mandarin (Citrus reshni) water-stressed seedlings required ABA accumulation in roots to induce ethylene synthesis (Gómez-Cadenas et al., 1996). Similarly, fruitlet abscission in ‘Satsuma’ mandarin (Citrus unshiu) has also been correlated to sugar shortage, leading to increased levels of ABA, which, in turn, triggered ethylene synthesis (Gómez-Cadenas et al., 2000).

In plants, ABA plays major roles in environmental stress responses. ABA biosynthetic pathway has already been completely elucidated in plants, and shown to be highly conserved in angiosperms (Xiong and Zhu, 2003). ABA signaling and action are, however, much less understood and, so far, only fragmentary information is available. ABA is synthesized from a C_{40} carotenoid precursor. In fact, many ABA-deficient mutants showed impaired carotenoid biosynthesis, as exemplified by Pinata, a sweet orange mutant which is defective in zetacarotene desaturase activity that leads to reduced ABA contents (Rodrigo et al., 2003). The first step of the pathway is the conversion of zeaxanthin into violaxanthin catalyzed by zeaxanthin epoxidase (ZEP), followed by conversion to neoxanthin, a 9-cis-epoxycarotenoid, in plastids. Then, 9-cis-neoxanthin undergoes an oxidative cleavage by 9-cis-epoxycarotenoid dioxygenase (NCED) resulting in xanthoxin, a C_{15} intermediate that is exported to the cytosol. NCED is the first committed enzyme to an ABA pathway and a major regulatory point. Xanthoxin is then attacked by a short-chain alcohol dehydrogenase/reductase (SDR), producing ABA-aldehyde. The last step in the pathway is the ABA-aldehyde oxidation to abscisic acid (ABA) by ABA-aldehyde Oxidase (AAO). AAO requires molybdenum as cofactor (MoCo), which is sulfurylated by MoCo sulfurase (Seo et al., 2000; Xiong et al., 2001; Xiong et al., 2002; Porch et al., 2006).

Several CitEST reads were strongly related to genes involved in ABA biosynthesis: zeaxanthin epoxidase (ZEP/ABA1/LOS6); 9-cis-epoxycarotenoid dioxygenase (NCED/NOT/VP14); abscisic aldehyde oxidase (AAO/SIT/TAO3); and molybdenum cofactor sulfurase (ABA3/LOS5/FLC) (Table S1). Recently, two NCED genes from Citrus sinensis have been cloned and characterized as presenting differential expression patterns and distinct enzymatic properties concerning substrate recognition: CsNCED1 was expressed in ripening fruits, whereas CsNCED2 transcripts were found only in chromoplast-containing tissues, such as flavedo (Rodrigo et al., 2006). ABA is catabolized by oxidation, reduction or conjugation (Cutler and Krochko, 1999). Phaseic acid (PA), dihydrophaseic acid (DPA), and glucose conjugates are the usual forms of inactivating ABA in plants. Recently, neophaseic acid (neoPA) was described as a novel ABA metabolite and detected in sweet orange fruits (Zhou et al., 2004). No reads from CitEST database matched Arabidopsis ABA-8’-hydroxylase CYP707A, the main enzyme involved in ABA inactivation (Saito et al., 2004).

ABA perception and signaling are still poorly understood. ROP10 is a small GTPase that participates in ABA signaling pathway as a negative regulator of ABA responses. It functions by modulating the expression of genes that respond to different ABA levels in Arabidopsis (Xin et al., 2005). We were able to identify two contig transcripts showing sequence similarity to ROP10 in the CitEST database.

ABA-responsive elements (ABRE) are present in promoters of ABA-regulated genes. ABRE-binding factors (ABF) activate transcription through the aforementioned cis elements by phosphorylation. TRAB1 from rice is a bZIP-domain transcription factor responsible for ABA regulation at ABRE (Kagaya et al., 2002; Kobayashi et al., 2005). CitEST database contained a single read sharing extensive sequence similarity to TRAB1. The role of TRAB1 in citrus remains unknown; however, the high level of sequence identity surpassed taxonomic classes (from a monocot to a dicot species), suggesting a conserved function.

OST1 (OPEN STOMATA1) is an ABA-activated serine-threonine protein kinase (AAPK) specifically implicated in the signaling pathway. Its expression is upregulated by ABA and osmotic stress. OST1 acts upstream to the production of reactive oxygen species (ROS), a key second messenger that induces cytosolic Ca\(^{2+}\) influx by activating plasma membrane Ca\(^{2+}\)-channels (Zhang et al., 2001; Mustilli et al., 2002). ABI1 and ABI2 are PP2C-type phosphatases that negatively regulate ABA activity in OST1-ROS cascade (Yoshida et al., 2006). Citrus transcriptome analysis identified OST1-related cDNAs, but none was highly similar to ABI1 or ABI2, suggesting that the OST1-ROS pathway may be present in citrus and ABI negative regulators may be absent from the database due to their low transcriptional activity in comparison to OST1. Alternatively, ABI activities may have been replaced by other proteins.

RCN1 (ROOTS CURL IN NPA1) is a serine-threonine phosphatase type 2 regulatory subunit that functions as a general positive regulator during early stages of ABA signaling, upstream of the cytosolic Ca\(^{2+}\) sensing (Kwak et al., 2002). Several transcripts sharing high sequence identity to RCN1 were identified in citrus. Seventeen reads were grouped into two EST contigs, demonstrating the extensive sequence conservation among citrus genomes.

Inactivation of RAC1 by ABA is critical for stomatal closure in Arabidopsis. RAC1/ROP6 is a small GTPase belonging to the Rho gene family, and associated to cytoskeleton regulation in yeast and animals (Lemichez et al., 2001). Transcripts showing sequence similarity to RAC1 were the most abundant of ABA metabolism and signaling-related genes identified in citrus transcriptome: 31 reads assembled in five contigs. As a central element in
plant adaptation to osmotic stress given its involvement in stomatal closure, it is reasonable to assume that RAC1 may play a strategic role in drought tolerance in citrus. GPA1 is a canonical Gα subunit of a heterotrimeric G protein implicated in downstream ABA signaling events (Wang et al., 2001). GCR1 is a G protein-coupled receptor, which physically interacts with GPA1, functioning as a negative regulator of GPA1-mediated ABA responses (Pandey and Assmann, 2004). In citrus transcriptome, we identified homologs of Arabidopsis GPA1 and GCR1, indicating a possible conserved mechanism in ABA signaling cascade involving these proteins. Since most investigations on ABA signaling have been carried out on model species, it is important to broaden these findings to other taxonomic groups to validate bona fide universal mechanisms of hormone action.

Auxin

We have identified 53 EST contigs showing similarity to proteins involved in auxin biosynthesis, metabolism, transport and signal transduction in the CitEST database. Results from comparisons against NCBI database can be found in Table S2. The longest contig consisted of 47 reads and showed similarity to a cullin-like protein. The contigs were assembled from sequences of almost all libraries but the most abundant were from leaf and fruit libraries (Table S2). The enzymes responsible for the biosynthesis of auxin are most active in young tissues: such as shoot apical meristems and in growing leaves and fruits. The same tissues are the locations where the highest concentrations of indole-3-acetic acid (IAA) are found. In fact, we have observed that the vast majority of sequences assembled in the contigs with similarity to proteins involved in IAA biosynthesis were from leaf (58.44%) and fruit (31.8%) libraries.

Auxins exert control over many important developmental processes in plants, including cell division and cell expansion, vascular tissue differentiation, root initiation, apical dominance, gravitropic and phototropic responses, flowering, fruit ripening, leaf senescence and abscission of leaves and fruit (Eckardt, 2001). Due to the importance of IAA in plant growth and development, extensive studies of the biosynthesis of this compound have been performed since its discovery as a plant hormone. The pathway for the biosynthesis of IAA in plants remains, however, to be elucidated, probably due to the existence of multiple pathways and possible functional redundancy among various participating enzymes. Indole-3-acetic acid is the most abundant naturally occurring auxin. Plants produce active IAA both by de novo synthesis and by releasining IAA from conjugates. This work emphasizes the analysis of the pathways involved in de novo IAA synthesis in citrus.

Genetic and biochemical experiments have demonstrated that two routes are responsible for IAA biosynthesis: a tryptophan-dependent and a tryptophan-independent one (Bartel, 1997). The starting point for IAA synthesis is in the tryptophan (Trp) biosynthetic pathway (Eckardt, 2001). Two routes from Trp to IAA are generally accepted as occurring in plants: one from Trp through indole-3-acetaldoxime (IAOx) and indole-3-acetonitrile (IAN) to IAA. Two Arabidopsis cytochrome P450 proteins, CYP79B2 and CYP79B3, can catalyse the conversion of Trp to IAOx in vitro (Hull et al., 2000). In addition to cytochrome P450s, several Arabidopsis proteins with similarity to flavin monoxygenases (FMOs) have been found to increase IAA production via a probable IAOx intermediate in superexpression mutants. The FMO encoded by YUCCA gene converts tryptamine to N-hydroxyl tryptamine (Zhao et al., 2001). Once YUCCA has converted tryptamine to N-hydroxyl tryptamine, another hydroxylation step is necessary to synthesize IAOx. This is presumably carried out by another FMO-like protein or a cytochrome P450. We have found five FMO contigs, from the largest number of reads (75). Six contigs of CYP83B1 were found and, according to Eckardt (2001), it is evidence that P450 CYP83B1 converts IAOx to its corresponding aci-nitro compound: the first step in indole-glucosinolate biosynthesis. This represents a metabolic branch point between IAA and indole-glucosinolate biosynthesis in Arabidopsis and CYP83B1 is found to play a role in regulating auxin homeostasis. One contig of CYP79B2 was found. In model systems, it catalyzes the formation of IAOx from Trp (Hull et al., 2000).

As mentioned earlier, it is often assumed that the conversion of IAOx to IAA proceeds through IAN. IAN can be converted to IAA through the action of nitrilases. The distribution appears to be limited to three families (Cruciferae, Graminae and Musaceae), although it is possible that similar enzymes catalyze this reaction in other plants. Three nitrilases from Arabidopsis (NIT1 to NIT3) are capable of converting IAN to IAA (Eckardt, 2001). In the present work, we have found two contigs of NIT4 that probably do not play a role in IAA biosynthesis, according to Piotrowski et al. (2000). We have also found one contig with 12 reads showing sequence similarity to NITRILASE1-like protein of Arabidopsis. In maize, two nitrilases ZmNIT1 and ZmNIT2 are expressed in seeds. ZmNIT2 efficiently hydrolyzes indole-3-acetonitrile to IAA and could thus be involved in auxin biosynthesis. However, some studies using nit1-1 mutants suggested that IAOx may be converted to IAA via a distinct route (Eckardt, 2001).

The alternative route proceeds from Trp via indole acetaldehyde (IAAld) to IAA. IAAld may be a good candidate for an intermediate between TAOx and IAA: instead of conversion to IAN, IAOx could be reduced to an imine followed by pH-dependent hydrolysis to IAAld, which can be converted to IAA via aldehyde oxidase (AO) (Normanly and Bartel, 1999). In fact, some investigations have shown that an AO, tentatively called IAAld oxidase, may be involved in IAA synthesis. In the present survey, we have
found two contigs with similarity to AO. According to our data, it seems that in citrus the same two vias are involved in the Trp-dependent IAA biosynthesis.

The versatility of auxins as signaling molecules is illustrated by the fact that a number of messages can be communicated simultaneously to different target cells. Precisely regulated auxin transport and redistribution have been implicated in some of these responses. Auxins are the only class of polarly transported phytohormones and their polar flow has been linked to many aspects of development, including establishment of the embryonic axis, continuous differentiation of vascular tissues and tropic growth responses, such as photo- and gravitropism. In higher plants, IAA is synthesized in shoot apices and transported towards the root tip, where it probably enters an opposite flow in the epidermis. The basipetal transport of IAA appears to be essential for the formation of continuous vascular strands and other aspects of cell polarity, while the opposite movement in the root epidermis is required for root gravitropism. Polar auxin transport proceeds in a cell-to-cell fashion involving influx and efflux through cell membranes. The directionality of the transport is thought to result from the polar distribution of specialized carrier molecules in the plasma membrane (Leyeser and Berleth, 1999). Members of the Arabidopsis PIN-FORMED1 (PIN1) and AUXIN-RESISTANT1 (AUX1) gene families are suspected to constitute components of the auxin efflux and influx machinery, respectively. The PIN1 protein has been implicated in general auxin transport along the apical-basal axis, evidenced by the impaired auxin transport in pin1 mutant stem segments. The recent identification of PIN1 gene product as a basically membrane-localized protein with structural similarities to bacterial membrane transporters strongly suggests a role in auxin efflux (Leyeser and Berleth, 1999).

In citrus, we have identified three contigs corresponding to PIN family members.

Auxin causes rapid changes in gene expression, and two families of proteins have been identified in this response: auxin response factors (ARFs) and Aux/IAA proteins (Callis, 2005). Auxin regulates a broad spectrum of developmental processes, mediating transcriptional regulation via protein degradation (Weijers and Jürgens, 2004). Regulation by proteolysis has emerged as a resounding theme in plant hormone signaling. The ubiquitin-mediated degradation of key regulatory proteins has been demonstrated, or at least likely, for all of the phytohormone response pathways (Smalle and Vierstra, 2004). In the case of auxin, the response pathway is normally subject to repression by a large family of transcriptional regulators called Aux/IAA proteins (Gray, 2004). These proteins dimerize with members of the AUXIN RESPONSE FACTOR (ARF) family of transcription factors, thus, preventing ARFs from activating auxin-responsive genes (Tiwari et al., 2004). Upon an auxin stimulus, an SCF ubiquitin ligase containing the TIR1 F-box protein ubiquitinates the Aux/IAA proteins, marking them for degradation by the 26S proteasome, thereby de-repressing the response pathway (Gray et al., 2001). Auxin de-represses the pathway by promoting AUX/IAA binding to SCF(TIR1) and leading to their degradation. SCF(TIR1) function requires AXR1-dependent RUB1 modification of an AtCUL1 subunit of the SCF. In citrus, we have identified three contigs corresponding to a putative CULLIN1 from A. thaliana. One is similar to a putative CULLIN3 from Oryza sativa (japonica cultivar-group), four contigs have similarity to TRANSPORT INHIBITOR RESPONSE1 (TIR1) from A. thaliana (Table S3). Seven ARF7, one ARF2 and one ARF1-like transcripts were also identified (Table S3). Eight Aux/IAA transcriptional regulators, four with similarity with auxin responsive family in Arabidopsis and four closer to Populus tremula, were identified in citrus transcriptome.

One transcript corresponding to ABP1 (AUXIN BINDING PROTEIN1) was identified in citrus species. The identification of a plant auxin-binding protein 20 years ago marked a major advance in understanding auxin perception in plants. Developing plants lacking ABP1 show defective cell elongation, fail to organize the basic plant body plan, and subsequently degenerate (Callis, 2005). But, it was demonstrated that the auxin-dependent SCF(TIR1) Aux/IAA interaction requires neither integral membrane proteins nor the candidate auxin receptor, auxin binding protein 1 (ABP1) (Weijers and Jürgens, 2004). Recently, TIR1 has been demonstrated to function as the auxin receptor in plants (Dharmasiri et al., 2005).

**Brassinosteroids**

We have identified 96 transcripts from citrus species that share sequence similarity to BR metabolism and signal transduction components from model species (Table S4, Table S5). Thirty-three are related to several steps of BR biosynthesis and processing, and the vast majority of them (20) are similar to BR oxidation P450 cytochrome proteins (Table S4). In citrus species, 63 ESTs are similar to BR signal perception and transduction: including 30 sequences homologous to receptor and receptor-like proteins, 25 similar to phosphorelay cascade and cross-talk intermediates and 7 whose deduced amino acid sequence is homologous to BR-induced transcriptional regulators (Table S5).

Brassinosteroids are C27, C28, and C29 steroids depending on their C-24 alkyl substituents, reviewed in Fujioka and Yokota (2003). In plants, they are present in a wide array of free, naturally occurring BRs. Brassinolide (BL) is the most biologically active C28 BR and, together with its C28 congeners, is widely distributed in the plant kingdom. BR biosynthetic pathway is highly networked and consists of several parallel BL pathways that branch and interact with each other (Noguchi et al., 1999). Initially, campesterol is converted to campestanol in an early C-22 oxidation pathway. Campestanol, in turn, is converted to castasterone (CS) through either early C-6 oxidation or late
C-6 oxidation, after which CS is further converted into BL. In citrus, we have identified homologs of the enzymes involved in the early C-22 oxidation pathway, such as DET2 and DWF4 and in both parallel C-6 oxidation pathways, including several CDP family members and BR6OX (Table S4). Thus, as demonstrated for rice, Catharanthus roseus, tobacco and Arabidopsis (Suzuki et al., 1995; Choi et al., 1996), BR biosynthetic pathways appear to be highly conserved in citrus species.

In plants, BRs are perceived by the transmembrane leucine-rich repeat (LRR) serine/threonine kinase protein BRI1 (BRASSINOSTEROID INSENSITIVE 1) that interacts with another LRR receptor kinase, BAK1 (BRI1-ASSOCIATED RECEPTOR KINASE 1) (Li et al., 2002; Nam and Li, 2002). Thus, BAK1 serves as a co-receptor for BRI1 to perceive the BR signal at the cell surface. The immediate targets for signal transmission from the receptor complex remain elusive, but several candidate signaling substrates have been identified for BRI1, including a thyrsothretin-like protein (TTL) and a TGF-β-receptor-interacting protein (TRIP1) (Nam and Li, 2004; Ehsan et al., 2005). TTL has been demonstrated to function as a negative regulator of BR-induced plant growth, although it fails to affect BR-induced transcriptional changes (Sablowski and Harberd, 2005). Alternatively, it is likely to function by locally regulating BR-induced responses, such as cell expansion (Nam and Li, 2004). The receptor kinase TRIP1 has been hypothesized to function as a general transcriptional regulator, although it remains to be demonstrated (Ehsan et al., 2005). The family of receptor kinase BR1 and BRI-like (BRL) is represented by 30 transcripts identified in C. sinensis, C. reticulata and P. trifoliata transcriptomes (Table S5). Similarly, 9 cDNAs correspond to the co-receptor BAK1 in citrus species (Table S5). We were unable to identify TTL and TRIP1 homologs in citrus using bioinformatic tools.

Cytoplasmic protein kinase BIN2 (BRASSINOSTEROID INSENSITIVE 2) functions downstream of the receptor complex to negatively regulate BR-initiated signal transduction and shares sequence homology to Drosophila SHAGGY kinase and mammalian glycogen synthase kinase 3 (GSK3) (He et al., 2002; Li and Nam, 2002). Three BIN2 homologs are present in citrus transcriptome and the deduced amino acid sequence of two of them is more than 50% identical to the sequence of Arabidopsis BIN2 (Table S5). BR-induced changes in gene expression have been demonstrated to involve BZR1 and BES1; there are two closely related nuclear proteins that function as positive transcriptional regulators (review in Li and Deng, 2005). In the absence of BR signaling, BZR1 and BES1 are present in phosphorylated forms, resultant from BIN2 kinase activity (Wang et al., 2002; Yin et al., 2005). Phosphorylated BZR1 has been hypothesized to be degraded by the 26S proteosome (Wang et al., 2002). BZR1 and BZL are members of a novel sub-family of bHLH transcription factors (He et al., 2005; Yin et al., 2005). The BZR1 binding site, CGTG(T/C)G, is found in four BR biosynthetic genes that are feedback regulated, including CPD and DWF4 (He et al., 2005). In citrus, BZR family is represented by 2 cDNAs containing the characteristic domain of unknown function found in the Arabidopsis family (Table S5). The phosphorylation status of BES1 is modulated by the nuclear serine/threonine protein phosphatase BSU1, which is also likely to be involved in the proteolytic degradation of BES1 via the Kelch-repeat domain at the N-terminus of the phosphatase (Mora-Garcia et al., 2004). C. sinensis and P. trifoliata BSU1 homologs show high sequence conservation at the kinase domain, although the identity at the Kelch repeats is less significant (data not shown). Citrus species have two BES-like proteins, sharing up to 40% of sequence identity between each other. The conserved domain of unknown function at the N-terminus of Arabidopsis BES1 is present in all identified citrus homologs. Thus, BR-regulated gene expression in citrus is hypothesized to be functionally equivalent to the Arabidopsis pathway.

Cytokinin

We have identified 44 ESTs corresponding to CK-related metabolism in citrus. From the total, 13 correspond to genes involved in the hormone biosynthesis, transport and catabolism (Table S6) and the remaining 31 are involved in CK-mediated signal transduction (Table S7).

The enzymes responsible for the first step in CK biosynthesis, the modification of the adenine moiety, are represented by one C. reticulata transcript highly similar to IPT3, IPT4, IPT7 and IPT8 from A. thaliana and several other species, and two C. sinensis cDNAs: one similar to IPT1 and one closer to IPT5 (Table S6). In general, IPT coding sequences are conserved throughout evolution, especially at the ATP/GTP binding motif (Kakimoto, 2001), thus the biological meaning of the family divergence in citrus species is likely to reflect functional redundancy. The enzymes modifying the adenine side-chain, the second step of CK biosynthesis, share extensive sequence similarity to several UDP-glycosyl transferases and corresponded to one EST singlet and one contig in citrus (Table S6). The importin permease family consists of several small hydrophobic polytopic membrane proteins involved in translocation of adenine-related compounds; however only two members, AtPUP1 and AtPUP2, have been demonstrated to translocate CK (Burkle et al., 2003). The family is represented in C. sinensis by two EST contigs that share sequence homology to both AtPUP1 and AtPUP2. In Arabidopsis, CK degradation is mediated by a five-gene family of cytokinin oxidases (CKX). Similarly in citrus, CKX-related transcripts were identified (Table S6).

Genetic and biochemical evidence has demonstrated that cytokinin signal transduction is primarily dependent on the bacterial and yeast two-component signal transduction pathways where stimuli-binding specifically leads to histi-
dine-asparagine multi-step phosphorelays, which in turn induce changes in gene expression (Grefen and Harter, 2004). Two-component signaling systems consist of sensor kinases, histidine phosphotransfer proteins and response regulators (Mizuno, 2005). The Arabidopsis cytokinin receptor kinases (Arabidopsis HISTIDINE KINASE2 (AHK2), AHK3, AHK4/CYTOKININ RESPONSE 1 (CRE1)/WOODENLEG (WOL)) contain a conserved extracellular cytokinin-binding domain called CHASE (cyclases/histidine kinases associated sensory extracellular), a histidine kinase and a receiver domain (Ferreira and Kieber, 2005; Riefler et al., 2006). In citrus, we have identified 5 EST contigs sharing extensive sequence conservation with Arabidopsis AHKs, including at the functional receiver domain (Table S7, Figure 2). Five Arabidopsis histidine-phosphotransfer proteins (AHPs) encode small proteins (of about 150 amino acids) mediating the phosphotransfer from the receptor kinases to the response regulators (Ferreira and Kieber, 2005).

The Arabidopsis genome has another 23 genes that are similar in sequence and domain structure to bacterial response regulators, and these encode both positive and negative elements in cytokinin signaling. In citrus, we have identified 3 transcripts corresponding to AHP genes from A. thaliana and from the woody model species Populus tremuloides (Table S7). Highest sequence homology is observed at the His-Asp phosphotransfer region, suggesting that these transcripts correspond to functionally active proteins in phosphorelay-mediated signal transduction (Figure 2). Arabidopsis RR genes belong to two main groups based on their sequence similarities: domain structure and transcriptional response to cytokinin (Mizuno, 2005). The type-A ARRs consist of a receiver domain and a short carboxyl terminus and their transcription is rapidly elevated in response to exogenous cytokinin; these are considered to be primary response genes (Liebfried et al., 2005; Kim et al., 2006). The type-B ARRs have a carboxy-terminal output domain that has a DNA-binding glutamic acid-rich protein (GARP) domain and a transcriptional activation domain in addition to the receiver domain, and in contrast, its transcription is not altered by cytokinin (Ferreira and Kieber, 2005; Mason et al., 2006). In citrus, we have identified 13 ARR genes: 7 corresponding to B-type and 6 to A-type response regulators.

Ethylene

The structural simplicity of the plant hormone ethylene contrasts with its dramatic effects on various developmental processes. These range from seed germination to senescence and organ abscission, and in the cellular processes that ethylene initiates in response to a diversity of environmental signals (Stepanova and Alonso, 2005).

Figure 2 - Cytokinin-mediated signal transduction in citrus. Schematic representation of AHK to AHP phosphorelay. Alignment of the receiver domain from A. thaliana and citrus AHK proteins and phylogenetic analysis of citrus AHKs. Alignment of the conserved histidine phosphotransfer domain from A. thaliana and citrus AHP proteins and phylogenetic analysis of the deduced amino acid sequence of citrus and Arabidopsis full-length proteins. Phylogenetic analysis was performed using full-length Arabidopsis proteins and the deduced amino acid sequence of citrus transcripts as described. Black and gray shading of amino acid residues represents sequence identity and similarity, respectively.
Ethylene is synthesized from the amino acid methionine via the intermediates S-adenosyl-L-methionine (AdoMet) and 1-aminoacyclopropane-1-carboxylate (ACC). The conversion of AdoMet to ACC is the first committed, and generally, rate-limiting step in ethylene biosynthesis and is catalyzed by the enzyme ACC synthase (ACS). Ethylene is then made from ACC by the enzyme ACC oxidase (ACO). Both ACS and ACO are encoded by multigene families in most plant species and these genes are regulated differently at the transcriptional level (Chae and Kieber, 2005). The *Arabidopsis* genome contains nine ACS genes that encode eight functional and one non-functional ACS protein. In *C. sinensis* transcriptome, we have identified three transcripts showing sequence similarity to *Arabidopsis* ACS enzymes (Table S8). Similarly, in *P. trifoliata*, the ACS family is represented by three transcripts (Table S8). Molecular genetic studies in *Arabidopsis* have provided evidence that ACS protein stability is regulated by the ubiquitin-26S proteasome, reviewed in Chae and Kieber (2005). The *Arabidopsis* genome contains nine ACS genes that encode eight functional and one non-functional ACS protein. In *C. sinensis* transcriptome, we have identified three transcripts showing sequence similarity to *Arabidopsis* ACS enzymes (Table S8). Similarly, in *P. trifoliata*, the ACS family is represented by three transcripts (Table S8). Molecular genetic studies in *Arabidopsis* have provided evidence that ACS protein stability is regulated by the ubiquitin-26S proteasome, reviewed in Chae and Kieber (2005). In this species, the gene ETO1 encodes a BTB (Broad-complex, Tramtrack, Bric-à-brac) domain-containing protein, a class that has been shown to link CUL3-based ubiquitin ligase to substrate proteins (Pintard et al., 2004). ETO1 also contains six predicted tetrapeptide repeat motifs, which are involved in diverse protein-protein interactions and can serve as a scaffold for the assembly of multiprotein complexes. In citrus, we have identified four ETO1 homologs (Table S8), including a transcript sharing more than 70% of sequence identity to the *Arabidopsis* protein.

Ethylene is sensed by a family of endoplasmic reticulum (ER)-localized membrane-bound receptors (Chen et al., 2002) that share sequence similarity with bacterial two-component histidine kinases (Stepanova and Alonso, 2005). The functional role of the kinase activity of ET receptors is not clear (Chen et al., 2005). The citrus ETR family consists of six transcripts, whose deduced amino acid sequence is highly similar to ETR proteins from model species, especially at the kinase domain (Table S9). The receptors function as negative regulators of the signaling partner CTR1 (Hua and Meyerowitz, 1998). CTR1 is a Raf-like protein kinase (Kieber et al., 1993) that has a role as a second negative regulator of the pathway that co-localizes and directly interacts with the receptors (Gao et al., 2003). We have identified several citrus cDNAs showing sequence similarity to CTR1 (Table S9). However, the sequence identity is mostly localized at the conserved Raf kinase domain. Thus, at this point, we cannot rule out a role for citrus CTR1-like transcripts in ET-independent signaling pathways.

EIN2, a novel plant-specific protein, is the downstream signaling partner of CTR1. EIN2 has two well-defined domains: (i) an N terminus with similarity to the NRAMP family of metal ion transporters and (ii) a unique hydrophilic C terminus (Alonso et al., 1999). Interestingly, we were unable to find EIN2 homologs in citrus transcriptome databases. In contrast, nine homologs of EIN3/EIN3-like proteins, a family of plant-specific transcription factors that are structurally and functionally conserved among several plant species (Stepanova and Alonso, 2005), were identified (Table S9). The activation of this family by ET is, at least in part, mediated by the regulation of their protein abundance through an ubiquitin-mediated proteasomal pathway. Two F-box proteins (EBF1 and EBF2) that form part of an SCF complex are involved in the regulation of EIN3 levels by ET in *Arabidopsis* (Guo and Ecker, 2003; Potuschak et al., 2003). Functional studies of EIN3 have demonstrated that it binds to the promoter sequences of an ethylene-inducible transcription factor gene ERF1, a member of the ethylene response element binding protein (EREBP) family of genes, reviewed in Chen et al. (2005). ET-triggered signal transduction controls the expression levels of a large number of target genes through what seems to be a transcriptional cascade (Alonso et al., 2003).

**Gibberellin**

The biosynthesis of the diterpene carboxylate hormone gibberellin (GA) has been well-characterized (Hedden and Phillips, 2000). We searched for genes involved in biosynthesis and signaling of GA in *Citrus sinensis*, and we were able to identify homologs to all biosynthesis components, and several transcripts shared sequence identity to genes involved in signaling (Table S10). At the first stage of GA biosynthesis, geranyl-geranyl diphosphate is cyclized to ent-kaurene by copalyl diphosphate synthase (CPS) and/or ent-kaurene synthase in the chloroplasts. Genes coding for these two enzymes share a high level of similarity, hindering identification. In the CitEST database, one homologous sequence to the genes coding for these enzymes was present as a singlet. In *Arabidopsis*, extremely low amounts of CPS mRNA were detected during plant development, with cell-specific expression (Olszewski et al., 2002). In the second step, ent-kaurene is oxidized by ent-kaurene oxidase (KO) to GA12-12-carboxylate by cytochrome P450 monooxygenases in the endoplasmic reticulum (Helliwell et al., 1998, Helliwell et al., 2001). Three EST-contigs similar to KO were identified, whereas three contigs and two singlets resembling KAO were identified. The conversion of GA12-12-carboxylate to C19-GA proceeds via the 13-hydroxylation pathway, resulting in GA20 and GA1, or alternatively by the non-13 hydroxylation pathway, which produces GA9 and GA4. These enzymes are encoded by a small multigene family in *Arabidopsis* (Olszewski et al., 2002). Transcripts for both enzymes were present in the citrus transcriptome: one contig showed low similarity to GA1 hydroxilase, and another to GA20 oxidase.
Gibberellins are synthesized in apical meristematic tissues and immature seeds, and have been detected in phloem translocation streams and in root exudates (Huntley et al., 2002; Lough and Lucas, 2006). Little is known about the regulation of GA catabolism, but the first step of GA degradation involves GA2-oxidases (GAXO) that hydroxylate C-2 of active GAs (Martin et al., 1999; Thomas et al., 1999, Sakamoto et al., 2001). Three contigs (two from C. sinensis) and one singlet showing homology to genes encoding for the GAXO group of enzymes were identified. GA2 oxidase, the enzyme involved in the first step of GA degradation, displayed high expression levels in all analyzed libraries (data not shown). GA2-oxidase genes have been cloned from several species, and in Arabidopsis they are encoded by a five-gene family, two genes associated with C19-GAs hydroxylation, and the remaining three coding for enzymes capable of hydroxylating C20 (Schomburg et al., 2003). The identified reads (26) were grouped into three distinct expression clusters with one containing 21 reads.

Regulation of GA biosynthesis is complex and it is likely to be feedback-regulated. Several important positive and negative regulators of GA signaling have been identified in model plants. GA is believed to bind a receptor, activating G-proteins, which in turn, enhance GA signaling pathways. GID1 was identified and characterized as a soluble receptor of GA in rice (Ueguchi-Tanaka et al., 2005). In citrus, two contigs with high similarity to GID1 were identified. The gene PHOR1 was described in Solanum tuberosum (Amador et al., 2001), and it has been shown that, upon GA binding, PHOR1 was translocated into the nucleus, where it acts as a positive regulator. No PHOR1 homologous sequences were found in the CitEST database.

The GA signal also activates protein kinase and GID2/SLY1-(F-box)-mediated degradation of DELLA proteins (Nambara et al., 1998). DELLA proteins function as negative regulators of GA signaling, and their degradation through the ubiquitin/proteasome pathway is considered a key event in the regulation of GA-initiated processes (Peng et al., 1997; Richards et al., 2001). One contig with high similarity to SLEEPY (SLY) protein was found in our analysis (Table S10). The expression pattern of the putative DELLA contigs in citrus displayed one read expressed in flowers, and all the others present in juvenile leaves. The observed profile of DELLA transcripts suggested that the genes present a tissue- and treatment-specific expression pattern. The limited number of reads prevented us from performing expression analysis in other citrus species. DELLA is a sub-family within the GRAS family of plant regulatory proteins involved in several aspects of plant development (Bolle, 2004). In rice, wheat, barley and maize, there is one homolog of Arabidopsis RGA/GAI coding for a DELLA protein repressor. However, in the dicot A. thaliana, five DELLA proteins were identified: RGA (REPRESSOR OF ga1-3) and GAI (GA INSENSITIVE) and three RGA-LIKE proteins: RGL1, RGL2, and RGL3. Recent evidence suggests that RGA, RGL1, and RGL2 are involved in modulating floral development in a tissue-specific manner (Lee et al., 2002; Wen and Chang, 2002; Tyler et al., 2004). In contrast, Lycopersicum esculentum genome appears to contain a single DELLA protein, named LeGAI (Bassel et al., 2004). Six DELLA-related contigs were identified in citrus. Four contigs were identified containing domains that allow the identification of GRAS sequences from the DELLA subfamily (Tian et al., 2004), suggesting that Citrus sinensis contains at least four DELLA genes. Incomplete consensus sequence in the majority of the contigs carrying the DELLA domain prevented us from performing a comprehensive phylogenetic analysis of the citrus DELLA family. Interestingly, three contigs presented C. sinensis reads matching contigs with reads from other species or genera (data not shown). Furthermore, the C. sinensis genome contains genes highly similar to other Citrus and Poncirus species.

The expression of Arabidopsis GAI in rice caused a dwarf phenotype, suggesting that GAI is sufficiently conserved to allow it to function in the context of a heterologous genome (Peng et al., 1999). Recent evidence demonstrates that GAI mRNA is translocated through the phloem (Haywood et al., 2005). A specific population of RNA molecules was identified in the phloem translocation stream (Yoo et al., 2004). Haywood et al. (2005) proposed that GAI mRNA delivery via phloem allows flexibility in fine-tuning developmental programs, allowing transcripts access to petal primordia and ground tissues.

DELLA proteins are targeted to o-GlcNAc modifications (Thornton et al., 1999). In Arabidopsis, SPINDLY (SPY) is a negative regulator of GA signal transduction. Sequence analysis of SPY suggests that it encodes an o-glucosyl-N-acetyltransferase, which can activate DELLA proteins (Jacobsen et al., 1996). In the citrus transcriptome, one contig and three singlets with high similarity to SPY were identified (Table S10). After degradation of DELLA proteins, positive transcription factors that were once blocked by DELLA, such as GAMYB, are free to activate the transcription of several genes (Gubler et al., 2002). A single sequence sharing similarity with GAMYB was found in Citrus reticulata. Achard et al. (2004) suggested that microRNA (miR159) modulates GA-mediated development via its effects on GAMYB activity, thus acting as a homeostatic regulator. We were unable to identify miR159 homologs in the CitEST database.

GA-mediated signal transduction is likely to encompass other genes, but the involvement of GAR2, SHI, GAST and several SCARECROW-regulators awaits further functional evidence. We have identified CitEST sequences homologous to those GA-signaling candidates; however, they were not referred to in this study due to space constraints.
Jasmonate

The jasmonate family of molecules includes jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA) and is derived from oxylipins, a group of biologically active compounds synthesized from the oxidative metabolism of polyunsaturated fatty acids (Schilmiller and Howe, 2005). These compounds are involved in regulating stress-induced gene expression, mechanical responses such as tendril coiling, and reproductive development (Browse, 2005).

In a survey of citrus transcriptome, we have identified 199 homologs of model plant genes involved in the metabolism and functioning as signaling partners of the jasmonate family of plant hormones (Table S11, Table S12). From the total, 87 citrus transcripts share sequence homology to JA biosynthesis components (Table S11), whereas the remaining 112 are similar to JA-triggered signal transduction pathway members (Table S12). Interestingly, the family of extensins, a group of homologous hydroxyproline-rich glycoproteins responsible for plant cell wall self-assembly and cell extension (Ringli et al., 2001) and JA-induced wounding and pathogen infection defense mechanisms (Shanmugam, 2005), is absent from citrus transcriptome databases. The crucial role of extensins in negative and positive regulation of cell expansion and elongation constitutes a major morphogenetic mechanism operating at all levels of plant growth and development, thus it is unlikely that this class of protein is absent from citrus species.

Most plant oxylipins are synthesized in a pathway initiated by lipoxygenase (LOX), a non-heme iron dioxygenase that adds molecular oxygen to either the 9 or the 13 position of the C18 chain of linoleic acid. Plants express nongenase that adds molecular oxygen to either the 9 or the 13 hydroperoxide reductase, and LOX itself (Schilmiller and Howe, 2005). Metabolism of 9-hydroperoxy linolenic acid (9-HPOT) to the jasmonate family of oxylipins by several enzymes, including closely related members of a cytochrome P450 family: allene oxide synthase (AOS), epoxy alcohol synthase, peroxigenase, alkyl hydroperoxide reductase, and LOX itself (Schilmiller and Howe, 2005).

The family of AOS enzymes transforms 13-hydroperoxy linolenic acid (13-HPOT) to the jasmonate family of compounds that includes JA/MeJA and their metabolic precursor, 12-oxo-phytodienoic acid (12-OPDA) (Schilmiller and Howe, 2005). Metabolism of 9-hydroperoxy fatty acids generates a group of oxylipins that are structurally related but distinct to the oxylipins generated by the 13-LOX pathway, the 9-hydroperoxy linolenic acid (9-HPOT). The 13- and 9-hydroxyperoxides are metabolized by allene oxide cyclase (AOC) isoforms (Schilmiller and Howe, 2005). AOC and AOS family homologs were identified in citrus species (Table S11). The α,β-unsaturated carbonyl moiety that distinguishes cyclopentenone oxylipins (e.g. 12-OPDA) from cyclosporinone oxylipins (e.g. JA) functions as a negative regulator of novel signaling activity (Seo et al., 2001).

The discovery of a gene encoding a JA carboxyl methyltransferase (JMT) indicates that MeJA is an important component of the mix of oxylipin signals that mediates defense responses (Seo et al., 2001; Zubieta et al., 2003). The deduced amino acid sequences of citrus JMT are moderately similar to the Arabidopsis protein with the highest similarity at the methyltransferase domain (Table S11). However, at this point a methyltransferase activity dissociated from JA metabolism cannot be ruled out. DADI is a member of DADI-like gene family in Arabidopsis and this family is hypothesized to regulate jasmonate production in response to other cues or to be involved in the biosynthesis of other classes of oxylipins (Matsui et al., 2004). In citrus, the family is represented by five transcripts including three C. reticulata-specific mRNAs (Table S11).

Jasmonates function as cellular regulators in diverse developmental processes, such as seed germination, root growth, fertility, fruit ripening, and senescence. In addition, JA activate plant defense mechanisms in response to insect wounding, various pathogens, and environmental stresses such as drought, low temperature and salinity and are also involved in the regulation of some stages of secondary metabolism (Cheong and Choi, 2003). The leucine-rich repeats and F-box motif protein COI1 is required to degrade a repressor of the jasmonate signaling pathway (Liecchi et al., 2006). Signaling in the jasmonate pathway depends on at least two massive signaling machines that interact in vivo to form a complex of at least 0.7 mDa (Feng et al., 2003). The first of these complexes is the SCFCOI1 complex, which is an E3-ubiquitin ligase (Devoto et al., 2002). In this complex, the F-box protein COI1 (Ren et al., 2005) physically associates with Skp-like proteins cullin and Rbx1 to form active SCFCOI1. The second multimolecular complex involved in JA signaling is the COP9 signalosome (CSN), which interacts in vivo with SCFCOI1 (Feng et al., 2003). We have identified citrus transcripts sharing extensive sequence conservation with COI1 (Table S12, Figure 3). The sequence identity is higher at the F-box domain (Figure 3). Interestingly, COI1-like transcripts are highly frequent in C. latifolia libraries.

Reversible protein phosphorylation has been demonstrated to be involved in JA signal transduction pathways leading to jasmonate-induced gene transcription (Jensen et al., 2002). A transposon-inactivation study revealed that mitogen-activated protein kinase 4 (MPK4) is required for jasmonate-responsive gene expression in Arabidopsis (Petersen et al., 2000). Interestingly, the deduced amino acid sequence of six citrus transcripts is more than 50% identical to AtMPK4 (Table S12), suggesting a functional conservation between the proteins from citrus species and their...
Arabidopsis counterpart. The other seven cDNAs from citrus present intermediate (from 44 to 28%) deduced amino acid identity to AtMPK4 (Table S12).

The expression of some JA-responsive genes is controlled in part by AP2/ERF-domain transcription factors (Yanhui et al., 2006). These proteins bind to “jasmonate- and elicitor-responsive elements” (JEREs) (van der Fits and Memelink, 2001). In citrus, several homologs of JA-induced transcriptional regulators were identified: including the development regulators AS1, ATAF2 and CPC (He and Gan, 2001; Delessert et al., 2005; Kwak et al., 2005); the basic-loop-helix JIN1 (Boter et al., 2004); the homeobox OCP3 (Coego et al., 2005); and the MYB telomere repeat-binding family of TRF1 and TRFL proteins (Yanhui et al., 2006) (Table S12). In citrus, JA-induced transcriptional regulators are highly frequent in biotic stress- and senescence-associated libraries, although distinct classes of factors show non-overlapping expression patterns (Figure 4).

Peptide hormones

Recently, a novel class of non-lipophilic peptide hormones was identified in several plant species, including A. thaliana, Solanum tuberosum and S. lycopersicum. Several secretary and non-secretary peptide signals have been demonstrated to be involved in plant growth regulation: including defense responses, callus growth, meristem organization, self-incompatibility (SI), root growth, leaf-shape regulation, nodule development, and organ abscission (Matsubayashi and Sakagami, 2006). These peptides have been identified by biochemical purification, genetic studies and in silico genome analysis. In general, they consist of a great number of highly-homologous genes that encode short open reading frames that are transcribed and translated at very low levels but severely affect plant development (Wen et al., 2004). In the CitEST database, we have identified 15 ESTs showing strong sequence similarity to previously characterized plant peptide hormones (Table S13). Interestingly, a large group of peptide hormones was absent from the screened citrus databases: including Solanum lycopersicum systemins (TomSys), S. lycopersicum and A. thaliana small, secreted, cysteine rich proteins (SCR/SP11, SCRL), A. thaliana inflorescence deficient in abscission (IDA) and IDA-like (IDL), A. thaliana POLARIS (PLS), A. thaliana CLAVATA3 and CLV3-like (CLV3, CLE and approximately another 100 similar sequences). Although this absence is unexpected, it might be attributed to the undetectable levels of expression of peptide hormone genes in wild type plants (Wen et al., 2004). In addition, the expression pattern of PSK1 and BRII1
SA biosynthetic pathway is derived from shikimic acid. 26 for biosynthesis and seven for its catabolism. The first Arabidopsis similarity to tome analyses, we have found 33 sequences with high et al. allel routes (Wildermuth, 1992a; Lee et al., 1995). In planta SA levels are probably the result of de novo synthesis (Yalpani et al., 1993); thus biosynthesis and metabolism knowledge functions as control steps for manipulating disease resistance.

In plants, the biosynthesis of SA consists of two parallel routes (Wildermuth et al., 2001). In citrus transcriptome analyses, we have found 33 sequences with high similarity to Arabidopsis components of SA metabolism: 26 for biosynthesis and seven for its catabolism. The first SA biosynthetic pathway is derived from shikimic acid. Firstly, chorismate is converted to prephenate, and prephenate to phenylpyruvate that is subsequently converted to phenylalanine by the action of the enzymes chorismate mutase (CM), prephenate dehydratase (PD) and phenylpyruvate amino transferase, respectively (Warpeha et al., 2006). Trans-cinnamic acid (CA) is synthesized from Phe by the action of Phe ammonia lyase (PAL). The production of benzoic acid (BA) from CA is not well understood and it could be step-limiting in SA biosynthesis. BA2H, the monooxygenase responsible for the conversion of BA to SA conjugates, is constitutively expressed in tobacco and is highly induced by the TMV inoculation in this species, just before the onset of HR-cell death, as well as under UV irradiation, or exposure to O3 (Leon et al., 1995, Chong et al., 2001). This step is also responsible for stress-induced flowering of Pharbitis nil (Hatayama and Takeno, 2003). BA2H is the first soluble cytochrome P450 identified in plants and animals, but no coding sequence is yet known in plants.

A second pathway for the synthesis of SA has been recently shown in plants (Wildermuth et al., 2001). Chorismate is converted to isochorismate by isochorismate-synthase (ICS) and then to SA and pyruvate, probably due to the action of pyruvate lyase (IPL). In citrus transcriptome survey, we have identified one transcript homologous to Arabidopsis ICSI gene covering the chorismate-binding region (Table S14). In model species, SAR responses require SA synthesized through ICSI. Interestingly, three of the four C. sinensis ESTs forming the contig C1-CR/CS originated from libraries of infected leaves.

In plants, SA is present as a free acid and as conjugated metabolites: SA 2-O-β-D-glucoside (SAG), glucosyl salicylate (GS) and methylsalicylate (MeSA) (Lee and Raskin, 1999). GS formation is transiently induced under pathogen attack and serves as a protective mechanism from the phytotoxic effects of high concentrations of SA (Lee and Raskin, 1999). In citrus transcriptome, four ESTs presented relatively high similarity to tobacco SA GTase. The methylation of SA into methyl-salicylate (MeSA) is mediated by a carboxyl methyl transferase that has salicylic acid (SAMT) as specificity of substrate. This enzyme belongs to a recently described family named ’SABATH’ with reference to model proteins (D’Auria et al., 2003). In citrus, we have identified four homologs to benzoic acid/salicylic acid:carboxyl methyl transferases (Table S14). The deduced amino acid sequences of these transcripts are highly conserved at the SA-binding critical residues in comparison to their tobacco counterparts (Zubieta et al., 2003).

We have found 70 homologs of genes involved in SA signaling pathway (Table S15). Plant-pathogen interactions trigger a complex network of regulatory processes among phytohormone-controlled pathways, including the antagonism between SA and JA signaling. In Arabidopsis, AtMPK4, WRKY70 and SSI2 have been demonstrated to have cross-talk intermediates and are discussed in JA subsection. Interestingly, ACD6 homologs were absent from citrus transcriptome. They encode a novel ankyrin repeat-

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**Figure 5** - Differential expression of peptide hormones in citrus species. The normalized number of reads for the transcripts in each library is represented in the y-axis. Citrus species libraries are represented by two-word abbreviations (CS, C. sinensis; CR, C. reticulata; CL, C. limonia; CG, C. aurantifolia; CA, Citrus aurantium; LT, C. latifolia; PT, Poncirus trifoliata). Hierarchical clustering of the expression patterns is represented by Roman numerals.
containing protein that participates in cell death control and probably has overlapping and/or redundant functions with other proteins. NDR1, a protein of unknown function, hypothesized to be a transducer of pathogen signals and/or to interact directly with pathogens, is represented by two homologous sequences in citrus EST databases.

Transcripts corresponding to the proteins forming the EDS1-PAD4-SAG101 complex were identified in citrus. These proteins present sequence similarity to acyl hydrolases, reinforcing the role of lipid signals in defense responses and indicating another probable cross-talk point in SA and JA/ET signaling (Wiermer et al., 2005). NPR1 and its homologous NPR4 are represented by two and seven homologs in citrus species, respectively. NPR1 plays a central regulator role in SA signaling and plant defense responses and NPR4 is required for the basal defense against pathogens.

Remarkably, we have found four sequences with high sequence similarity to SABP2 in citrus transcriptome. In Arabidopsis, it displays SA-stimulated lipase activity essential for basal resistance (Kumar and Klessig, 2003; Wiermer et al., 2005). It has been thought to be the long-sought-after SA receptor; however, recent evidence has implicated it in the hydrolysis of inactive MeSA to SA (Forouhar et al., 2005). More interestingly, citrus SABP2 displays higher levels of sequence conservation to a C. sinensis transcript coding for an ethylene-induced esterase (Zhong et al., 2001). This suggests the existence of a direct link between SA- and ET-mediated signaling pathways.

The SA-responsive genes are mainly regulated by a set of transcription factors that includes TGA, WRKY70 and WHIRLY1, which are present in citrus transcription databases. TGA constitutes a conserved plant subfamily of NPR1-regulated basic domain/Leu zipper (bZIP) transcription factors associated with detoxification and defense (Klinedinst et al., 2000; Pontier et al., 2001). The most important NPR1-interacting TGA factors are TGA2 and TGA3 that are represented by several transcripts in citrus. In Arabidopsis, WHY1 is dependent on SA but it functions in an NPR1-independent manner to induce plant defense gene expression and to mediate SAR (Desveaux et al., 2004). This type of regulator response confirms that SA signaling has an NPR1-independent branch. The great similarity observed between the citrus and Arabidopsis transcripts probably reflects functional conservation between the species. Another interesting finding is the high sequence conservation observed between VAD1 and two citrus transcripts. VAD1 is hypothesized to represent a new function in cell-death control associated to cells in the vicinity of vascular bundles. It contains a GRAM domain that functions in membrane-associated processes to protein or lipid binding (Lorrain et al., 2004).

Concluding Remarks

This preliminary survey of citrus components of hormone-associated pathway has provided useful information for further studies of developmental control in these spe-

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**Figure 6** - Schematic representation of citrus hormone signaling pathways controlling stress and developmental responses. Phytohormones are represented by black circles and pathway components are shown in white boxes. Full lines represent genetic and direct interactions and dashed lines show hypothesized interactions. Arrowheads demonstrate positive interactions; lines without arrowheads are for interactions where directionality is unknown and blocked lines represent negative interactions. For clarity reasons, not all relevant genes or interactions have been shown.
cies. It has allowed the identification of conserved members of signaling pathways in a non-model species and the elaboration of a framework for future studies (Figure 6). The results obtained here indicate high conservation in hormone biosynthetic pathways between model species and citrus. Signaling pathways are generally less conserved, although the vast majority of investigated processes were identified in citrus species (Figure 6). These studies will help to elucidate the molecular basis of developmental control and to understand how environmental factors modulate plant development and the expression of phenotypic characters. The results obtained give a new perspective on several aspects of hormonal regulation of physiological processes in citrus.

Acknowledgments

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References


Hormones in citrus


Kim HJ, Ryu H, Hong SH, Woo HR, Lim PO, Lee IC, Sheen J, Nam HG and Hwang I (2006) Cytokinin-mediated control of leaf longevity by AHK3 through phosphorylation of...
α subunit GPA1 and regulates abscisic acid signaling. Plant Cell 16:1616-1632.


Protein Families (Pfam), http://www.sanger.ac.uk/Software/Pfam/ (October 15, 2006).

Supplementary Material

The following online material is available for this article:
Table S1
Table S2
Table S3
Table S4
Table S5
Table S6
Table S7
Table S8
Table S9
Table S10
Table S11
Table S12
Table S13
Table S14
Table S15
Supplemental References
Figure S1
Figure S2
This material is available as part of the online article from http://www.scielo.br/gmb.

Associate Editor: Marco Aurélio Takita
Table S1– Citrus ESTs homologous to *Arabidopsis* and *Oryza sativa* functionally-characterized ABA biosynthesis and signaling pathway genes.

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<th>References</th>
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Identity percentage at the amino acid level.

GenBank accession number.
Table S2 – Citrus ESTs homologous to *Arabidopsis* and *Oryza sativa* functionally-characterized auxin metabolism genes.

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**a**Gene name abbreviations: AAO: aldehyde oxidase, CYP: cytochrome P450, NIT: nitrilase, YUCCA: yucca tree.


**c**Identity percentage at the amino acid level.

**d**CYP79B2 family in *A. thaliana*: AT4G31500, AT4G39950.

**e**NIT family in *A. thaliana*: AT3G44310, AT3G44300.

**f**YUCCA family in *A. thaliana*: AT1G04180, AT1G04610, AT1G21430, AT2G33230, AT4G13260, AT4G28720, AT5G11320, AT5G25620, AT5G43890.
Table S3 – Citrus ESTs homologous to *Arabidopsis* functionally-characterized auxin signaling pathway genes.

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**BIG**  
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80.0  
0.0  
Zinc finger ZZ type,  
Polar auxin transport  
Gil *et al.*, 2001

**CUL family**  
AT4G02570  
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89.0  
0.0  
Cullin domain, component  
Gray *et al.*, 1999;  
Quint *et al.*, 2005

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Auxin transport protein,  
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Identity percentage at the amino acid level.

Protein motif: SCF: SKP1/Cullin/F-box protein.

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AUX/IAA family in *A. thaliana*: AT5G65670, AT5G57420, AT5G43700, AT4G32280, AT4G29080, AT4G28640, AT4G14550, AT3G62100, AT1G04100, AT1G04240, AT1G04250, AT1G04550, AT1G15050, AT1G15580, AT1G151950, AT1G52830, AT1G80390, AT2G01200, AT2G22670, AT2G33310, AT2G46990, AT3G04730, AT3G15540, AT3G16500, AT3G17600, AT3G23030, AT3G23050.

CULIIN family in *A. thaliana*: AT1G02980, AT1G26830, AT1G69670.

PIN/PID family in *A. thaliana*: AT1G23080, AT1G70940, AT1G77110, AT2G01420, AT2G34650, AT5G15100, AT5G54490.
Table S4 – Citrus ESTs with homology to genes involved in brassinosteroid metabolism in *Arabidopsis thaliana*.

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Identity percentage at the amino acid level.

Table S5 – Citrus ESTs with homology to genes involved in brassinosteroid-initiated signal transduction in *Arabidopsis thaliana*.

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<sup>c</sup> % identity.

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transcription factors
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*Identity percentage at the amino acid level.*

Table S6 – Citrus ESTs with homology to genes involved in cytokinin metabolism and transport in *Arabidopsis thaliana*.

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**Catabolism**
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cIdentity percentage at the amino acid level.
Table S7 – Citrus ESTs with homology to genes involved in cytokinin signal transduction in *Arabidopsis thaliana*.

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response regulators

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<sup>b</sup>Identity percentage at the amino acid level.

Table S8 – Citrus ESTs with homology to genes involved in ethylene biosynthesis in *Arabidopsis thaliana*.

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\(^a\) Protein motifs: Domain architectures and functional motifs detected using Pfam, InterPro, and SMART databases.

\(^b\) ESTs: Expressed sequence tags.

\(^c\) Identity: Percentage identity of Citrus EST sequences to the *Arabidopsis thaliana* homologues.

\(^d\) ACO family: ACO family of aminocyclopropane-1-carboxylate oxidase.

\(^v\) ACS family: ACS family of 1-aminocyclopropane-1-carboxylate (ACC) synthase.
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cIdentity percentage at the amino acid level.


eACO family: AT2G19590, AT3G47190.

fACS family: AT1G01480, AT1G62960, AT2G22810, AT3G49700 (ETO3), AT4G08040, AT4G11280, AT4G26200, AT4G37770, AT5G1690, AT5G65800, AT3G47190.
Table S9 – Citrus ESTs with homology to genes involved in ethylene signal transduction in *Arabidopsis thaliana*.

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<td>Ecker, 1990; Alonso et al., 1999</td>
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**Primary transcription factors**

**Secondary transcription factors**
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- **CTR**: constitutive triple response; **EBF**: ethylene binding factor; **EIL**: EIN3-like; **EIN**: ethylene insensitive; **ERS**: ethylene response sensor; **ETR**: ethylene receptor; **HLS**: hookless; **RAN**: responsive to antagonist.
- **C**: contig; **CG**: *Citrus aurantifolia*; **CR**: *Citrus reticulata*; **CS**: *Citrus sinensis*; **PT**: *Poncirus trifoliata*; (number of reads).
- **Identity percentage at the amino acid level.**
- **EIN3/EIL family**: AT5G21120, AT1G73730.
Table S10 – Citrus ESTs with homology to genes involved in gibberellic acid biosynthesis, metabolism and signal transduction in *Arabidopsis thaliana*.

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<sup>a</sup> Name of the EST

<sup>b</sup> EST name

<sup>c</sup> Percentage identity
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*C: contig, CL: Citrus limonia, CR: Citrus reticulata, CS: Citrus sinensis, LT: Citrus latifolia, PT: Poncirus trifoliata, (number of reads).*

*Identity percentage at the amino acid level.*
Table S11 – Citrus ESTs with homology to genes involved in jasmonic acid biosynthesis in *Arabidopsis thaliana*.

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<td>oxide cyclase, formation of 9-hydroxyperoxyde metabolism</td>
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Matsui et al., 2004

Chloroplast phospholipase A1, 2004

Oxylipin biosynthesis

AMP-dependent synthetase and ligase, fatty acid esterification, 2006

Liechti et al., 2006
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|   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| JMT | AT1G19640 | C7-CS/CR (53) | 45.5 | 9e-89 | s-adenyl-methione-dependent | Seo et al., 2001; |
| C5-CS (2) | 35.4 | 2e-46 | carboxyl methyltransferase, formation of methyljasmonate | Zubieta et al., 2003 |
| C4-CS (4) | 33.2 | 8e-30 | from jasmonic acid |
| C6-CR (2) | 30.8 | 1e-32 |
| C1-CS/CR (2) | 30.1 | 6e-40 |
| C2-CS/CR (11) | 30.1 | 2e-33 |
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*b*Identity percentage at the amino acid level.


*Functional domain abbreviations: AMP: adenosine monophosphate; CYP: cytochrome protein; LH: leucine and histidine; NADH: reduced nicotinamide adenine dinucleotide.*
Table S12 – Citrus ESTs with homology to genes involved in jasmonic acid-mediated signal transduction in *Arabidopsis thaliana*.

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Tsuchiya et al., 1999
Ellis and Turner, 2001
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Grant et al., 2000

Santamaria
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Guan et al., 2005

Yoshida et al., 2002

Lukowitz et al., 2001

Zabala et al., 2005

Devoto et al., 2002

León et al., 1998; Sun et al.
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Identity percentage at the amino acid level.

Functional domain abbreviations: CUC: cup-shaped cotyledons, HAD: haloacid dehalogenase, LRR: leucine-rich repeat, MAP: mitogen-activated protein, MYB: retroviral oncogene v-myb, NAC: Petunia NAM and Arabidopsis ATAF1, ATAF2, and CUC2, NAM: no apical meristem, SCF: Skp1, Cdc53, an F-box complex, WWE: domain is named after three of its conserved residues (tryptophan, tryptophan and glutamate), WRKY: a 60 amino acid region that is defined by the conserved amino acid sequence WRKYGQK at its N-terminal end, together with a novel zinc-finger-like motif.
Table S13 – Citrus ESTs homologous to functionally-characterized plant peptide hormones.

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PT11-C1-900-089-E02-CT  63.9  1e-99  localization, BR signal
CR05-C3-702-027-G11-CT  63.1  5e-90  transduction, systemin receptor
CS00-C3-700-068-G06-CT  57.1  3e-88
CR05-C1-103-056-H03-CT  42.5  1e-73

**ROT4/DVL**  AT2G36985
CS00-C3-703-011-C08-CT  25.6  3e-12  RTF (ROTUNDIFOLIA)  Narita *et al.*, 2004; Wen *et al.*, 2004
LT33-C1-003-050-C02-CT  22.2  2e-05  domain - 29-amino acid domain, whole protein 54 amino acids in length

*Database searches of CitEST have been unable to recover homologs of the following peptide hormones: *Solanum lycopersicum* systemins (TomSys), *S. lycopersicum* small, secreted, cysteine rich proteins (SCR/SP11), *Arabidopsis thaliana* small, secreted, cysteine rich proteins-like (SCRL), *A. thaliana* inflorescence deficient in abscission (IDA, AT1G68765), *A. thaliana* inflorescence deficient in abscission-like IDL (AT3G25655), *A. thaliana* POLARIS (PLS) (AT4G39403), *A. thaliana* CLAVATA3 (CLV3, AT2G27250), *A. thaliana* CLV3-like (CLE, AT1G73165 and approximately other 100 similar sequences).*

*Gene name abbreviations: PSK1: phytosulfokine; RALFL: rapid alkalinization factor-like; BRI1: brassinosteroid insensitive1; ROT4/DVL: rotundifolia4/devil.*


*Identity percentage at the amino acid level.*
Table S14 – Citrus ESTs with homology to genes involved in salicylic acid metabolism in plants.

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<th>Arabidopsis thaliana</th>
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**Gene name abbreviations:**

- **CM**: chorismate mutase
- **ICS**: isochorismate synthase
- **PAL**: phenylalanine ammonia-lyase
- **AmSAMT**: *Antirrhinum majus* S-adenosyl-L-methionine:salicylic acid methyltransferase
- **NtSAGTase**: *Nicotiana tabacum* salicylic acid glucosyltransferase

**C**: contig, **CA**: *Citrus aurantium*, **CG**: *Citrus aurantifolia*, **CL**: *Citrus limonia*, **CR**: *Citrus reticulata*, **CS**: *Citrus sinensis*, **LT**: *Citrus latifolia*, **PT**: *Poncirus trifoliata*, (number of reads).

**Identity percentage at the amino acid level.**
Table S15 – *Citrus* ESTs with homology to genes involved in salicylic acid signal transduction in plants.

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CA26-C1-002-088-C11-CT  51.1  2e-45
CG32-C1-003-085-F02-CT  54.7  1e-43
LT33-C1-003-064-B03-CT  74.3  1e-96

ICS1  AT1G74710  C1-CR/CS (4)  69.1  5e-72  isochorismate synthase activity,  Wildermuth et al., 2001
salicylic acid biosynthesis,  systemic acquired resistance

NDRI  AT3G20600  C1-CS (3)  50.9  8e-52  defense response to pathogenic bacteria and fungi,  Coppinger et al., 2004
CR05-C3-701-043-E04-CT  43.2  4e-21  incompatible interaction,
located in membrane

NPR1  AT1G64280  C9-CG/CS/CA (5)  58.9  2e-82  cell death, response to heat,  Cao et al., 1997
C10-CR (2)  54.1  4e-91  response to bacteria and insect,  systemic acquired resistance,
salicylic acid mediated signaling pathway

NPR4  AT4G19660  C11-CS/CR/LT (5)  46.8  5e-95  response to pathogenic bacteria,  Liu et al.,
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<td>PT11-C2-300-016-C11-CT</td>
<td></td>
<td>5e-39</td>
<td>implicated in the cross-talk</td>
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<td>NtCAT-1</td>
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<td>catalase activity; serves to protect cells from the toxic effects of</td>
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<td></td>
<td>P49319</td>
<td>e-163</td>
<td>hydrogen peroxide; inhibited by salicylic acid</td>
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</table>

Chen et al., 1993

2005
<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
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<th>E-value</th>
<th>Score</th>
<th>Function Description</th>
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<td><strong>NtSABP2</strong></td>
<td>AAR87711</td>
<td>C1-CG/CR/CS (7)</td>
<td>56.0</td>
<td>3e-81</td>
<td>lipase, alpha/beta hydrolase, fold, may generate a lipid-derived signal, responsible for the conversion of MeSA into SA</td>
<td>Kumar and Klessig, 2003; Forouhar et al., 2005</td>
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<td>the conversion of MeSA into SA</td>
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<td><strong>PAD4</strong></td>
<td>AT3G52430</td>
<td>C16-CS (2)</td>
<td>43.6</td>
<td>2e-59</td>
<td>response to insect, systemic acquired resistance, salicylic acid mediated signaling defense response to pathogenic bacteria, incompatible interaction, leaf senescence; protein binding; has lipase activity</td>
<td>Jirage et al., 1999</td>
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<td>AT5G14930</td>
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<td>Feys et al.,</td>
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<td>transcription factor with a basic region leucin zipper, DNA binding</td>
<td>Johnson et al., 2003</td>
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<td>TGA family putative membrane-associated protein containing a GRAM domain, a lipid or protein binding signaling domain</td>
<td>Johnson et al., 2003</td>
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Database searches of CitEST have been unable to recover homologs of the ACD6, a novel ankyrin repeat and transmembrane-domain containing protein.


Identity percentage at the amino acid level.