Gene expression analysis in Citrus reveals the role of gibberellins on photosynthesis and stress

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Running title: Effect of GA on Citrus Transcriptome

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ABSTRACT

The effect of gibberellins (GA) on internode transcriptome was investigated in transgenic Carrizo citrange (*Citrus sinensis* x *Poncirus trifoliata*) plants overexpressing endogenous *CcGA20ox1* (encoding a GA biosynthetic gene), and in non-transformed explants treated with GA_3, using a citrus cDNA microarray. Substantial modulation of gene expression was found in sense *CcGA20ox* plants. Extensive upregulation of genes involved in photosynthesis and carbon utilization, and downregulation of those involved in protein synthesis and ribosome biogenesis was shown for the first time in plants with higher GA content. Importantly, increase of net photosynthesis in attached leaves was also demonstrated. Expression of other genes belonging to functional groups not reported previously to be regulated by GA (mainly abiotic and biotic stresses, and cuticle biosynthesis), and genes involved in cell division and cell wall architecture were also differentially expressed. Culture of citrus explants for 24 h in GA_3 solution produced much lower changes in the transcriptome compared to *CcGA20ox* plants (1.6% vs 16%, respectively, of total genes in the microarray), suggesting that most of the changes observed in *CcGA20ox* plants were a consequence of long-standing GA effect. Interestingly, genes related to abiotic and biotic stresses were similarly modulated in transgenics and GA_3-treated explants.

*Key-words***:** abiotic stress; biotic stress; carbon utilization; citrus; gibberellin; microarray; photosynthesis; protein synthesis; ribosome biogenesis; transgenics

INTRODUCTION

Gibberellins (GAs) are plant growth regulators that control various aspects of growth and development. GAs are tetracyclic diterpenoids synthesized from geranylgeranyl diphosphate by three groups of enzymes: cyclases, cytochrome P450-dependent monooxygenases, and 2-cetoglutarate-dependent dioxygenases (Sponsel & Hedden 2004). Three kinds of enzymes are included in the latter group, GA 20-oxidases (GA20ox) and GA 3-oxidases (GA3ox), that act consecutively to produce the bioactive GAs (GA_1 and GA_4), and the inactivating GA 2-oxidases (GA2ox). Most of the genes encoding enzymes of GA metabolism have been isolated in many species (Sponsel & Hedden 2004). In the case of dioxygenases they constitute small gene families whose enzymatic activity plays an important function in the homeostatic regulation of active GA levels. Overexpression and down-regulation of *GA20ox* gene in Arabidopsis (Coles et al 1999), hybrid aspen (Eriksson et al 2000), tobacco (Vidal et al 2001), cultivated apple (Bulley et al 2005) and, more recently, the citrus rootstock Carrizo citrange (Fagoaga et al 2007) alters the concentrations of bioactive GAs and the phenotype, indicating that the regulation of this gene is crucial in modulating GA flux in the late stages of the pathway.

With the advent of microarray technology it is now possible to examine changes in transcript abundance for thousands of genes within a single experiment. However, very little work of this kind is found in the literature using transgenics with modified GA metabolism. For instance, transcriptome analysis of developing xylem of transgenic hybrid aspen plants overexpressing Arabidopsis *AtGA20ox1* has been reported (Israelsson et al 2003). In this case, the highest transcript changes occur in genes generally restricted to the early stages of xylogenesis (including cell division, early expansion and late expansion), associated with the higher xylem fiber development of

the transgenics. However, this transcriptome study examined only a selected group of genes since xylem-biased cDNA microarray was utilized. While data from microarray experiments have the potential to add substantial knowledge to our understanding of how genes are regulated in response to GAs, only few microarrays studies in Arabidopsis during seed germination (Ogawa et al 2003; Yamauchi et al 2004) and in rice callus (Yazaki et al 2003) and seedlings (Yang et al 2004) in response to GA application have been reported.

Carrizo citrange is used largely as a rootstock in citrus due to its resistance to *Citrus tristeza virus* and Phytophthora spp. and to the high fruit quality and yield that provides to the scion (Saunt 2000). Therefore, the genetic manipulation of this rootstock is of great interest with the purpose of modifying the growth of the scion and to facilitate diverse cultural practices. Transgenic plants of Carrizo citrange overexpressing an endogenous *GA20ox* gene (*CcGA20ox1*) (Vidal et al 2003) have been recently produced (Fagoaga et al 2007). The sense plants contain more GA_1 (the active GA in citrus) in developing shoots, and display a phenotype characterized mainly by longer internodes and thorns and reduced leaf size and thickness.

Over the past years, the Citrus Functional Genomic Project (CFGP) in Spain has generated useful tools for citrus research, such as a collection of ESTs from large-scale cDNA sequencing using cDNA libraries derived from a wide range of tissues, developmental stages, and biotic and abiotic stress conditions. With the purpose of carrying out functional analysis of the citrus transcriptome, 12672 probes corresponding to 6875 putative unigenes have been spotted on glass slides (Forment et al 2005).

In this work, with the aim of better understanding the role of GAs on vegetative growth in citrus, we carried out large-scale gene analysis in internodes of Carrizo citrange from: a) transgenic plants overexpressing sense *CcGA20ox1* (*CcGA20ox*

plants) and b) explants after short-term GA_3 application, using the citrus cDNA microarray previously described.

MATERIALS AND METHODS

Plant material and hormonal treatments

Plants of the transgenic sense S23 line of Carrizo citrange (a *Citrus sinensis* L. Osb. x *Poncirus trifoliata* L. Raf. hybrid), showing a clear phenotype and significantly higher active GA_1 concentration (Fagoaga et al 2007), produced from rooted stems and grown in soil under natural ambiance conditions, in an insect-proof greenhouse, were used in the experiments. Plants bearing an empty vector were used as control. Entire young developing shoots (about 10 cm long) from autumn flush were collected, and the corresponding internodes, including the nodes, used for transcriptome analysis.

To investigate short term GA effect, young shoots 10 cm long bearing 5-6 developing leaves were excised from Carrizo citrange wild type plants, about 3 month-old, multiplied from seeds. The explants were incubated at 24ºC and 16 h light/ 8 h dark in vials containing 40 mL of 10 μM GA_3 in Murashige and Skoog medium starting 2 h after beginning the light period, and internodes were sampled at times 0 h and 24 h for transcriptome analysis.

RNA isolation

Total RNA was extracted according to Malmberg et al (1985). For microarray experiments, the RNA was additionally cleaned up with the RNeasy Plant Mini Kit (Qiagen). For semiquantitative RT-PCR analysis, RNA was treated with RNase-free DNase (Qiagen) to remove genomic contamination. Total RNA was quantified using a NanoDrop ND-100 Spectophotometer (NanoDrop Technologies).

Preparation of labelled cDNA probes

RNA (30 μg) was reversed transcribed using 400 units of SuperScript III reverse transcriptase (Invitrogen) in the presence of aa-UTP (334 μM), 6 μg oligo(dT) 24-mer, 500 μM each dATP, dCTP and dGTP, 166 μM dTTP and 10 μM DTT in the provided buffer (final volume 30 μL) for 3 h at 50ºC. Aminoallyl-labelled cDNA was treated with 0.25 M NaOH, purified in a Qiaquick column (Qiagen). and resuspended in 10 μL $NaHCO_3$ 100 mM, pH 9. cDNA was post-labelled with Cy3 or Cy5 CyDye NHS-ester (Amersham), and purified using a Qiaquick PCR purification kit (Qiagen). The samples were quantified in a NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies).

Microarray hybridization, data acquisition and data analysis

Gene expression analysis was conducted using a citrus cDNA microarray containing 12672 probes corresponding to 6875 putative unigenes (Forment et al 2005). A recent assembly using all the 85965 ESTs obtained in the Citrus Functional Genomics Project until now indicates that the whole collection includes 27551 unigenes and that the cDNA microarray used actually contains 6034 unigenes.

Mycroarray hybridization and scanning was carried out as described elsewhere (Forment et al 2005) with some modifications. Labelled cDNA from experimental (labelled with Cy5) and control (labelled with Cy3) samples (about 60 pmoles) were dried separately and resuspended in fresh hybridization solution [containing 50% (v/v) formamide]. Samples were boiled for 1 min before using for hybridization at 42ºC. Microarray slides were scanned with a GenePix 4000B (Axon Instruments) using GenePix 6.0 image acquisition after discarding non-homogeneous and aberrant spots. Data were transformed using an intensity-based Lowess function (Yang et al 2002) with Acuity 4.0 software (Axon Instruments) and analyzed only for features with no missing values or with only one missing value from the three replicates. Identification of differentially expressed genes was done by Student's *t* test corrected for multiple testing

using the Benjamini-Hochberg method (Benjamini and Hochberg 1995). Genes that satisfied the statistical threshold (adjusted *p* values < 0.05) and at least a 1.6-fold change in expression were identified as differentially expressed in *CcGA20ox* plants.

To identify differentially expressed genes in response to short-term GA_3 application, significance analysis of microarrays (SAM; Tusher et al 2001) was conducted on the four independently normalized data sets using two-class unpaired analysis with 100 permutations. Significant genes, with an estimated false discovery rate (FDR) of less than 5% and a 1.6-fold expression cutoff, were identified.

Citrus unigenes functional annotation

Unigenes functional annotation was performed using EST2uni (http://bioinf.comav.upv.es/est2uni) carrying out BLASTX against the UniRef90 non-redundant protein database (http://www.ebi.ac.uk/uniref) and the full set of Arabidopsis proteins provided by TAIR, using default parameters and arbitrary non-stringent threshold of 10^{-5} for *E*-value. Unigenes were annotated with the description of the most similar UniRef90 cluster of proteins or, when no significantly similar UniRef90 cluster was found, with the description of the most similar Arabidopsis protein, if any. Functional motifs were also identified by using a HMMER search (Eddy 1998) against the pfam database (http://pfam.janelia.org). For citrus unigenes functional analysis and discussion the corresponding most similar Arabidopsis protein was always used.

Microarray functional analysis

Genes found to be differentially expressed were classified by functional categories using FunCat version 2.1 (http://mips.gsf.de/projects/funcat) program. This allowed a broad functional classification of the upregulated or downregulated genes. FatiGO program (http://fatigo.bioinfo.ochoa.fib.es) was then used to look for functional enrichment of GO terms over-represented in a particular set of genes relative to a

reference group. We used only the subset of genes in the genome that are present on the citrus microarray as the reference.

Semiquantitative RT-PCR analysis

Total DNase-treated RNA (3 µg) was denatured and reverse transcribed using a First Strand cDNA synthesis kit (Amersham Biosciences). Aliquots of cDNA solutions (1 µL) were used in PCR reactions (50 µL final volume) in the presence of 0.6 µM of each clone-specific primer (Supplemental Table I) and 2.6 units of Expand High Fidelity enzyme (Roche). PCR reaction parameters were 5 min at 95ºC, a variable number of cycles (depending on the gene, to get exponential amplification; Supplemental Table I) of 30 s at 95ºC, 45 s at 60ºC and 45 s at 72ºC, and a final extension of 10 min at 72ºC. Aliquots of PCR products (20 µL) were separated by electrophoresis on a 1% (v/v) agarose 1xTAE gel and stained with ethidium bromide before quantifying using the Gene Snap (SynGene) program. Citrus *actin* was used as a constitutive control.

Photosynthetic measurements

Net photosynthetic rate (Pn) was measured on fully expanded leaves randomly selected of control plants and transgenic Carrizo citrange plants overexpressing *CsGA20ox1* gene using a portable photosynthesis system CIRAS-2 (PP Systems). Pn was measured at CO_2 concentration of 824 ± 7 ppm and a photosynthetically active radiation (PAR) of 600, 800 and 1000 µmol m^{-2} s^{-1} (higher concentration of CO_2 than in normal atmosphere was used because carboxylation by Rubisco is not saturated at the current CO_2 concentration; Drake et al, 1997). The flow rate of air through leaf chamber was 195 mL min^{-1}, and air temperature maintained at 25-27ºC.

Statistical analysis of net CO_2 fixation data was made using SPSS statistical software (Norusis 1993). Two-way ANOVA was carried out to determine the effect of genotype and interactions between genotype and PAR supply.

RESULTS

Overexpression of *CcGA20ox1* modifies transcript level of endogenous *CcGA20ox1*

First, we confirmed that the transgenic Carrizo citrange line S23 used in this work contained high levels of *CcGA20ox1* transgene transcripts (about 11-fold compared to control plants) (Supplemental Figure 1A). On the other hand, endogenous *CcGA20ox1* transcripts, analyzed by RT-PCR using a 3'-region sequence not included in the transgene, were not detected in S23 (Supplemental Figure 1B), probably as a result of the negative feed-back regulation mechanism showed previously to regulate transcription of that gene (Vidal et al 2003). The same kind of plant material employed in this analysis was used to measure global gene expression changes in the selected transgenic lines.

Overexpression of *CcGA20ox1* causes substantial remodelling of the transcriptome

Three biological replicates, each consisting of three internodes from young developing shoots of the representative sense S23 line, were used for transcriptome analysis. Internodes from shoots of plants bearing an empty vector were used as control. Box plots of the log2 ratios after Lowess normalization (Yang et al 2002) for each of the 3 replicates showed fairly similar data spreads (Fig. 1A), revealing a good reproducibility. The upper left and right squares of the Volcano plot [that represents, for each gene in the microarray, the log of the ratio between transgenic and control expression versus the log of probability (*p* value) that the observed ratio occurs at random] contained a fairly high number of spots (Fig. 1B), meaning that many significantly differentially expressed genes were present in *CcGA20ox* plants.

In *CcGA20ox* plants, 1228 ESTs corresponding to 726 unigenes (12% of total unigenes in the microarray) were differentially expressed (*p* value of a Student's *t*-test corrected by Benjamini-Hochberg < 0.05). This means that genetic modification of GA

metabolism resulted in a large remodelling of the transcriptome. 336 of the differentially expressed genes (46.3% of the total) were upregulated (Supplemental Table II), and 390 (53.7% of the total) downregulated (Supplemental Table III). This suggests that both upregulation and downregulation play a similar role in the response to *CcGA20ox1* overexpression. Interestingly, 14.6% of these differentially expressed genes (106) did not show significant similarity with any Arabidopsis protein, and probably includes citrus-specific genes involved in pathways or functions specific to citrus.

An analysis of sequence similarity was performed for annotation of differentially expressed unigenes (Supplemental Tables II and III). For functional analysis and discussion of citrus unigenes, the corresponding most similar Arabidopsis protein was always used. Arabidopsis protein similarity could be assigned to 85.4% of the differentially expressed sequences (620 genes). To get an integrated view of gene expression changes and to explore the biological processes in which the differentially expressed genes are involved, their functional role was examined using the MIPS (Munich Information Center for Protein Sequences; http://mips.gsf.de) Functional Catalogue (FunCat; Ruepp et al 2004) for the corresponding most similar Arabidopsis protein (see Supplemental Table IV for upregulated and Supplemental Table V for downregulated genes). In most categories, similar proportions of genes were found in both cases (Table I). However, a bias toward upregulation was observed for energy (4.4% vs. 1.8%) and interaction with the cellular environment (7.0% vs. 3.9%) categories. On the other hand, a bias toward downregulation was observed for protein synthesis (1.4% vs. 7.9%).

Results from microarray analysis were confirmed by PCR monitoring six genes selected randomly according to their microarray expression profiles and putative

functions [five upregulated: photosynthesis (RuBisCO small subunit and RuBisCO activase), abiotic stress (RD22), cell wall metabolism (XTH) and one with no annotation available; one downregulated: secondary metabolism (GGPS)] in control and *CcGA20ox* plants. Sequence identifiers and number of ESTs corresponding to each gene are given in the legend of Fig. 2. Transcript levels (Fig. 2A) showed good correlation with gene expression changes detected by microarray studies (R = 0.96 between these two methods; Fig. 2B), supporting the results obtained with the transcriptome approach.

Specific functional gene classes are enriched in *CcGA20ox* plants

To assist in identifying key processes that were altered in *CcGA20ox* plants we looked for functional enrichment in the differentially expressed set of genes using FatiGO tool corresponding to the most similar Arabidopsis protein (Al-Shahrour et al 2004), based on Gene Ontology (GO) terms (Ashburner et al, 2000). GO categories identified as significantly over-represented in the upregulated set (Fig. 3A, Supplemental Table VI) were 'photosynthesis, light harvesting' (GO:0009765, $p = 0.03$) and 'carbon utilization by fixation of carbon dioxide' (GO:0015977, $p = 0.01$), while in the downregulated set we found those of 'protein biosynthesis' (GO:0006412, $p = 4 \cdot 10^{-9}$) and 'ribosome biogenesis' (GO:0007046, $p = 1 \cdot 10^{-4}$) (Fig. 3B, Supplemental Table VII). The GO categories corresponding to 'response to water' (GO:0009414, $p = 0.0677$) and 'cuticle biosynthesis' (GO: 0042335, $p = 0.0677$) (Supplemental Table VI), although not significantly over-represented, are also included in Fig. 3A because they will be subject of discussion later on.

Diverse genes within the light harvesting (e.g. chlorophyll a/b binding proteins) and light electron transfer reactions (e.g. ferredoxin and photosystems I and II), as well as in the generation of precursor metabolites and energy (e.g. glycolate oxidase, fructose-biphosphate aldolase, thioredoxins and ferredoxins) and carbon utilization (e.

g. carbonic anhydrases, RuBisCO small subunits and glyceraldehyde 3-phosphate dehydrogenases) categories were found to be upregulated (Fig. 3A, Supplemental Table VI). Since the category of 'carbon utilization by fixation of carbon dioxide' was significantly over-represented we wanted to know whether genes of the Calvin-Benson cycle, in addition to those already present in the enriched categories, were also upregulated. Most of the pentose phosphate (Calvin-Benson) cycle genes (Fig. 4) were upregulated, including RuBisCO (2.7 fold, *p*-value< 0.05), GADPH (2.6 fold, *p*-value <0.05), fructose-bisphosphate aldolase (3.9 fold, *p*-value <0.05), and fructose-bisphosphatase (1.9 fold, *p*-value <0.05). Genes encoding phosphoribulokinase (3.7 fold, *p*-value 0.059) and transketolase were also upregulated (1.5 fold, *p*-value <0.05) although they did not fulfil our threshold values. In addition, upregulation of two citrus genes encoding RuBisCO activase, that regulate the activity of RuBisCO (4.8 fold, *p*-value <0.05) was found in *CcGA20ox* plants. Possible expression changes of other genes in the pathway (encoding phosphoglycerate kinase, sedoheptulose-bisphosphatase, and ribulose-5-phosphate isomerase) could not be assessed because they were not represented in the cDNA microarray. These results strongly suggest that overexpression of *CcGA20ox1* induced an increase of carbon fixation capability in transgenic plants.

The 'protein biosynthesis' and 'ribosome biogenesis' categories mainly included genes encoding ribosomal proteins (at least 25 and 15 belonging to 60S and 40S ribosomal subunits, respectively) (Fig. 3B, Supplemental Table VII). Three elongation factors were also found in the 'protein biosynthesis' category.

Transcriptional activation of photosynthesis-related genes led to increased photosynthetic capacity of *CcGA20ox* plants

The observation that there was an overall upregulation of genes encoding proteins of the photosystems and chlorophyll binding proteins (Fig. 3A, Supplemental Table VI), as well as of genes of the carbon fixation pathway (Fig. 4), prompted us to hypothesize if this could be evidence of increased photosynthesis capacity in transgenic plants. To confirm this, net CO_2 uptake was measured in leaves of *CcGA20ox* and control plants. The net photosynthetic CO_2 uptake in young leaves of *CcGA20ox* plants was significantly higher than in control plants at photosynthetic active radiation (PAR) of 800 and 1000 μmol m^{-2} s^{-1} (Table II). No significant differences were found at 600 μmol m^{-2} s^{-1} (Table II). Overexpression of *CcGA20ox1* also increased significantly both stomatal conductance (g_s) and transpiration rate (E), regardless of PAR value (Table II). However, water use efficiency, estimated as Pn/E ratio, was similar in control and *CcGA20ox* plants. Only at PAR 1000 this parameter was slightly lower in *CcGA20ox* plants.

Overexpression of sense *CcGA20ox1* produces upregulation and downregulation of specific genes

It was of interest to examine differentially expressed genes in *CcGA20ox* plants (Supplemental Tables II and III) not included in the enriched categories described before, with the purpose of identifying metabolic and physiological processes possibly related to *CcGA20ox* plants phenotype. In this analysis we have concentrated in genes involved in cell division and wall metabolism, stress, and those encoding transcription factors related to these processes.

Cell division and cell wall biosynthesis and modification

Since *CcGA20ox* plants show increased cell divisions in the elongating internodes (Fagoaga et al, 2007), it was expected that they show altered levels of transcripts genes involved in the cell cycle regulation and cell wall biosynthesis and modification. Two

genes encoding cell division proteins were upregulated while a cyclin-dependent kinase (CDKB2;2), regulating normal cell cycle progression in Arabidopsis (Andersen et al 2008) was downregulated (Supplemental Table VIII). Upregulation of an endo-1,4-β-D-glucanase (required for normal cellulose formation), a cellulose synthase-like (involved in the synthesis of matrix polysaccharides; Cosgrove, 2005), four ß-1,3-glucanases (which brake down callose, a polymer abundant in the cell plate of dividing cells; Scheible and Pauly, 2004), and three xyloglucan endotransglucosylase-hydrolase (XTH) genes (encoding enzymes that regulate cell-wall extensibility) was also observed (Supplemental Table VIII).

Abiotic and biotic stress

There are reports in the literature suggesting that GAs may be involved in different kinds of abiotic stresses (Achard et al 2006; Magome et al 2004). Since the GO categories 'response to water' and ´cuticle biosynthesis´ (a process known to be altered by drought; Aharoni et al 2004) were close to be significantly over-represented in the upregulated set of genes (Fig. 3A, Supplemental Table VI), we looked for genes involved in abiotic stress (desiccation, osmotic, salt and oxidative) which were differentially expressed in *CcGA20ox* plants (Table III). We found that 23 genes (15 of them related to response to water) were upregulated and 11 downregulated. Upregulation of genes encoding LEA5, dehydrins, delta 1-pyrroline-5-carboxylate synthase (P5CS) and cysteine proteinases (RD19, RD21) was observed in *CcGA20ox* plants. Plant surfaces are protected by the cuticle, a complex lipid structure composed of an outermost epicuticular wax layer overlying a cuticle membrane layer, which provides a protective barrier against environmental stress, mainly drought, and pathogens (Chen et al 2003). In the case of *CcGA20ox* citrus plants, we found that in addition to those of water response many genes involved in lipid (e.g. GDSL-motif lipase/hydrolases and

acyl-ACP thioesterase), wax (*WAX2*) and cuticle biosynthesis (e.g. Very Long Chain Fatty Acid Condensing Enzyme and lipid transfer proteins) were also upregulated (Supplemental Table IX), suggesting that in those plants there was probably cuticle modification to prevent water loss. Interestingly, diverse kinds of genes related to biotic stress (e.g. six chitinases and ten Kunitz protease inhibitors) were downregulated in *CcGA20ox* plants (Supplemental Table III).

Transcription factors

Transcription factors constitute a substantial fraction of all eukaryotic genomes, and serve to integrate expression of genes with particular environmental and developmental stimuli (Riechmann & Ratcliffe 2000). Table IV summarizes the expression profiles of citrus genes that encode putative transcription factors showing differential expression in *CcGA20ox* plants, 11 of them upregulated and 8 downregulated. Interestingly, many of these transcription factors seem to regulate processes related to phenotypic alterations found in *CcGA20ox* plants (e.g. change in plant architecture, stress resistance). For instance, within the former, we found those encoding the BEL1-like homeodomain 1 (BLH1) (a mutation of this gene causes a dwarf phenotype in Arabidopsis; Bhatt 2004), a WRKY (many WRKY have a regulatory function in the response to pathogen infection and other stresses; Eulgem et al 2000), a myb (MYB52, shown to respond to ABA; Yanhui et al 2006), a bHLH (bHLH062, which responds to diverse kinds of stresses; Heim et al 2003) and a YABBY (the rice *YAB1* gene, very similar to that of citrus, is involved in the feedback regulation of GA biosynthesis; Dai et al 2007). Within the downregulated transcription factors there was a high mobility group B protein (HMGB) (whose overexpression in Arabidopsis alters seedling growth under various stress conditions; Kwak et al 2007), a NAC transcription factor (RD26) (involved in desiccation response in Arabidopsis; Yamaguchi-Shinozaki et al 1992) and

members of myb (CCA1 and MYB78 and MYB121, which respond to salt stress and ABA stimulus, respectively in Arabidopsis; Yanhui et al 2006).

Short term application of GA_3 alters transcriptome of explant internodes

To better understand how GAs control vegetative citrus shoot development we also analyzed how the transcriptome was altered after short-term GA_3 application. Internodes from growing shoots, at the same developmental stage as those used for transgenic analysis, were cultured in the presence of 10 μM GA_3, collected 6, 12 and 24 h later and used for RT-PCR analysis. After 24 h a clear reduction of *CcGA20ox1* transcript level took place (data not presented), meaning that GA_3 had been efficiently transported and acted metabolically in these GA_3-treated explants. For this reason, internodes at 0 h and after 24 h, untreated and GA_3-treated (four biological replicates, 5 shoots per replicate) were used for transcriptome analysis. RNA corresponding to 0 h was used as reference material.

We identified 123 ESTs as significantly regulated in response to GA_3 treatment which corresponded to 75 unigenes, 26 upregulated and 49 downregulated (Supplemental Table X). FatiGO analysis was also performed but no enriched GO category was found (data not shown). However, differentially expressed genes were classified into MIPS categories with the purpose to understand their possible biological function (Supplemental Table XI for upregulated and Supplemental Table XII for downregulated genes).

As occurred in *CcGA20ox* plants (Supplemental Table III), genes involved in protein synthesis (two encoding ribosomal proteins and one encoding a translation iniciation factor) were also downregulated after short-term GA_3 application (Supplemental Table X), supporting the idea that GAs may induce differential protein translation. However, in contrast to *CcGA20ox* plants, we could not detect an increase

in RuBisCO transcripts 24 h after GA_3 application using citrus explants cultured *in vitro*, neither any other gene involved in carbon fixation.

The most striking result obtained with GA_3-treated internodes was that most of the differentially expressed genes were involved in abiotic and biotic stress (Supplemental Table X), an effect which was also apparent in *CcGA20ox* plants. In the case of upregulated genes they included genes (14 out of the 22 differentially expressed) encoding proteins involved in water response (dehydrins and cysteine proteinase), temperature perception and response (inositol-3-phosphate synthase and omega-3 fatty acid desaturase), and biotic stimulus and plant defense responses (osmotin-like proteins). In addition, genes corresponding to two groups of proteins involved in plant defense response (basic endochitinases and Kunitz family proteins) were downregulated in GA_3-treated explants.

DISCUSSION

The increase of GA content, produced by overexpression of an endogenous *GA20ox*, induced a large transcriptome rearrangement in citrus internodes. This genetic manipulation of GA metabolism was associated with global upregulation of genes involved in photosynthetic and carbon utilization, an effect described for the first time in plants with elevated GA content. In contrast, lower changes in gene expression were observed in the internode transcriptome of citrus explants after 24 h of GA_3 application, suggesting that short-term GA_3 application has no effect on photosynthesis in citrus. Therefore, the transcriptional activation of photosynthesis related genes in *CcGA20ox* plants may be the result of long-term adaptation to altered GA content. Expression of some of the genes involved in carbon fixation has been reported previously to be altered by GA_3 application. For instance, the levels of RuBisCO subunits in broad bean and soybean leaves increases after 1 h of GA_3 treatment as a result of translation (Yuan &

Xu 2001). Transcript levels of fructose-bisphosphate aldolase also increases in rice roots within 24 h of GA_3 application (Konishi et al 2004). Carbonic anhydrase (that catalyses the reversible hydration of CO_2 and thus the availability of CO_2 to RuBisCO) increases in *Brassica juncea* leaves upon GA_3 treatment (Hayat et al 2001). Net CO_2 fixation in leaves of transgenic citrus *CcGA20ox* plants was higher at PAR values (800 and 1000 μmol m^{-2} s^{-1}) similar to those found in the field under a sunny day. This supports the conclusion that, although we do not have data on biomass production, the global upregulation of genes corresponding to be over-represented GO categories of 'photosynthesis' and 'carbon utilization' in *CcGA20ox* plants has a physiological effect. This may be related to the more compact mesophyll (palisade and spongy parenchyma layers) tissue in the transgenic leaves of citrus plants overexpressing *CcGA20ox1* (Fagoaga et al 2007). Interestingly, in tobacco, a positive effect of GA on net photosynthesis was observed when measured on entire plants overexpressing *GA20ox*, but not when measured on single leaves (Bielmelt et al 2004). In this case, however, mesophyll of transgenic plants was not apparently different from wild-type. The effect of GA on photosynthesis has been controversial, some authors finding that GA_3 application has a positive (Yuan & Xu 2001; Hayat et al 2001), negative (Dijkstra et al 1990) or no effect (Cramer et al 1995). These apparently contradictory results may be due to the different experimental systems and methods used by the different authors to determine photosynthesis (Nagel & Lambers 2002).

The unexpected result that extensive downregulation of genes corresponding to "ribosome biogenesis" and "protein biosynthesis" functional categories occurred both in *CcGA20ox* plants and GA_3-treated cuttings suggests that the entire protein synthesis machinery was affected as an early effect of GA action. However, the decreased expression of these genes may not affect the synthesis of all proteins because at least

those involved in photosynthesis and carbon fixation probably increased in transgenic plants. This downregulation may be rather to the result of a change in the pattern of protein synthesis, as reporter earlier in GA_3-treated barley aleurone (Jacobsen & Beach 1985).

We found that diverse genes encoding proteins involved in cell division and cell wall metabolism were altered in *CcGA20ox* plants. This result was expected considering that GAs induce cell elongation and/or division in internodes of diverse species, and cell division in internodes of *CcGA20ox* plants (Fagoaga et al 2005). Positive effect of GA_3 on cyclin-dependent kinases (Sauter 1997) and XTH activity and transcription (Potter and Fry 1993; Jan et al 2004), related to internode elongation, has been reported.

Expression changes of genes involved in water stress mitigation were found in *CcGA20ox* plants, an effect not reported previously in transgenic plants with modified GA metabolism, as well as in GA_3-treated cuttings. This may have a preemptive function by checking the expected increased water usage requirements as a result of enhanced GA growth. It has been demonstrated that transcript accumulation of some of these genes [for instance members of dehydrin and LEA family in rice (Xu et al 1996), sunflower (Cellier et al 1998), barley (Zhu et al 2000) and wheat (Lopez et al 2001) and of delta 1-pyrroline-5-carboxylate synthase in tobacco (Kavi Kishor et al 1995)] increases drought tolerance. Thus, our results suggest the possibility that *CcGA20ox* plants may also display higher water stress tolerance. In support of this idea, genes encoding wax and cuticle biosynthesis (which might produce cuticle modification and so prevent water losses) were also upregulated in *CcGA20ox* plants. Similarly, in *Populus* response to water stress is associated with upregulation of GO categories not only of 'response to water' but also of 'wax biosynthesis' and 'cuticle biosynthesis' (Street et al 2006). However, this conclusion must be further substantiated by carrying

out direct water stress experiments using citrus *CcGA20ox* cuttings. On other hand, the downregulation in *CcGA20ox* plants and GA_3-treated cuttings of several genes involved in pathogen defense response (Kim et al 2003) suggests that GA overproduction may reduce this kind of protection.

The nuclear-localized DELLA proteins are negative regulators of GA signal transduction which are degraded by the action of GAs (Fleet & Sun 2005). It has been proposed that DELLA are also integrators of responses to environmental signals in Arabidopsis (Achard et al 2006; Achard et al 2008; Navarro et al 2008). According to this idea, we can speculate that over-representation of water response genes and differential expression of other biotic and abiotic genes may be the consequence of a decrease in DELLA protein caused by elevated GA levels. Nevertheless, that hypothesis has to be confirmed by testing the expression of DELLA genes in *CcGA20ox* plants.

In summary, the increase of GA content produced by overexpression of an endogenous *GA20ox* induced global upregulation of genes involved in photosynthetic and carbon utilization and overall downregulation of genes involved in protein biosynthesis and ribosome biogenesis, effects described for the first time in plants with elevated GA content. This genetic manipulation of GA metabolism was also associated with an increase of net CO_2 fixation capacity. Genes related to diverse abiotic (mainly water response and cuticle biosynthesis) and biotic stresses were also differentially expressed both in the transgenic citrus and in GA_3-treated cuttings, although the possible physiological meaning of these changes must be further substantiated.

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FIGURE LEGENDS

Figure 1. Global expression changes in Carrizo citrange internodes overexpressing *CcGA20ox1*. A. Box plots displaying the intensity log-ratio distribution after Lowess normalization procedure for each of the three replicates (R1, R2, R3). B. Volcano plots for test of *CcGA20ox1* overexpression effect. X-axis shows relative expression between transgenic and control samples averaged across three replicates. Y-axis shows the significance of gene-specific *t*-test. The horizontal line represents the $p = 0.05$ threshold and the vertical bars represent 2-fold change.

Figure 2. Confirmation of microarray data by RT-PCR. A. Expression of six differentially expressed genes in *CcGA20ox* plants by semiquantitative RT-PCR. aCL8Contig9 (9 ESTs), no annotation available; aCL172Contig2 (6 ESTs), RD22; aCL48Contig1 (2 ESTs), RuBisCO activase; aCL3307Contig1 (2 ESTs), xyloglucan endotransglucosylase/hydrolase; aCL43Contig3 (11 ESTs), RuBisCO small subunit; aCL960Contig1 (1 ESTs), geranylgeranyl pyrophosphate synthase; actin (CX289161; used as internal control). The three lanes under each genotype correspond to three biological replicates. B. Correlation between RT-PCR and microarray analysis for six differentially expressed genes in *CcGA20ox* plants. Values are means from three biological replicates ± SE.

Figure 3. Hierarchical view of Gene Ontology (GO) Biological Process categories significantly over-represented with upregulated (A) and downregulated (B) genes obtained using FatiGO tool. Significant categories (*p* value from Fisher's exact test corrected for multiple hypothesis testing < 0.05) are shown using a colour scale according to their significance level. Other categories required to complete the hieratchy are shown in grey.

Figure 4. Changes in transcript levels of genes involved in the Calvin-Benson cycle in internodes of *CcGA20ox* plants. Dark grey boxes correspond to mean values of upregulated genes (at least 1.6 fold change and $p < 0.05$). RuBisCO, aCL43Contig3 (13 ESTs) and aCL43Contig4 (2 ESTs); RuBisCO activase, aCL48Contig1 (2 ESTs) and aCL48Contig2 (10 ESTs); GADPH, aCL642Contig2 (2 ESTs); Fructose-bisP aldolase, aCL73Contig1 (2 ESTs); Fructose-bisphosphatase, aCL3697Contig1 (1 EST); Transketolase, aCL2018Contig1 (3 ESTs); Phosphoribulokinase, aCL1319Contig1 (2 ESTs).

Supplemental Figure 1. Transcript levels of CcGA20ox1 transgene (A) and of endogenous *CcGA20ox1* (B) in three biological replicates of representative sense (S23), and control (C) lines. Transcripts were determined by semiquantitative RT-PCR as described in Materials and Methods, using a Citrus *actin* (CX289161) as an internal control.

Supplemental Table I. Primers used for semiquantitative RT-PCR analysis.

Supplemental Table II. Citrus gene annotations of upregulated genes in *CcGA20ox* plants.

Supplemental Table III. Citrus gene annotations of downregulated genes in *CcGA20ox* plants.

Supplemental Table IV. Classification of upregulated genes in *CcGA20ox* plants into functional categories according to MIPS.

Supplemental Table V. Classification of downregulated genes in *CcGA20ox* plants into functional categories according to MIPS.

Supplemental Table VI. Gene ontology biological processes categories over-represented in the upregulated set of genes in *CcGA20ox* plants.

Supplemental Table VII. Gene ontology biological processes categories over-represented in the downregulated set of genes in *CcGA20ox* plants.

Supplemental Table VIII. Differentially expressed genes involved in cell division and cell wall biosynthesis and modification.

Supplemental Table IX. Differentially expressed genes involved in fatty acid and lipid pathways.

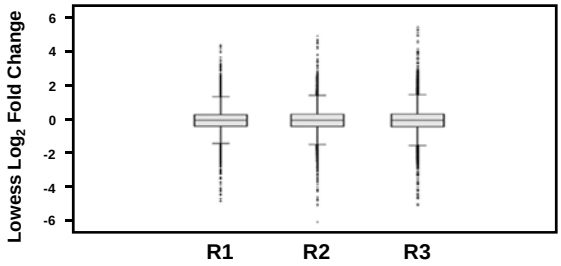
Supplemental Table X. Differentially expressed genes after GA_3 application.

Supplemental Table XI. Classification of upregulated genes after GA_3 application into functional categories according to MIPS.

Supplemental Table XII. Classification of downregulated genes after GA_3 application into functional categories according to MIPS.

Figure 1

A


Lowess Log2 Fold Change
6
4
2
0
-2
-4
-6
R1
R2
R3

B

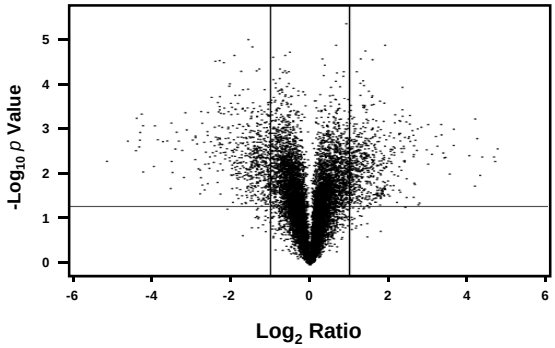
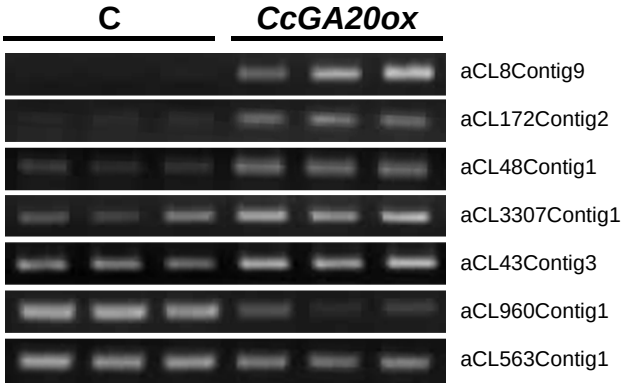

-Log10 p Value
5
4
3
2
1
0
-6
-4
-2
0
2
4
6
Log2 Ratio

Figure 2

A


C
CcGA20ox
aCL8Contig9
aCL172Contig2
aCL48Contig1
aCL3307Contig1
aCL43Contig3
aCL960Contig1
aCL563Contig1

B

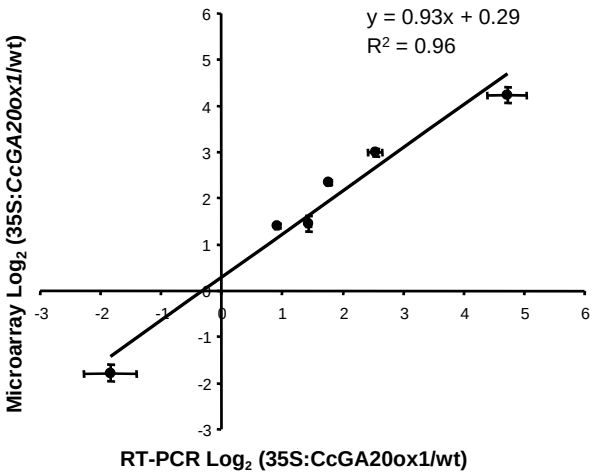
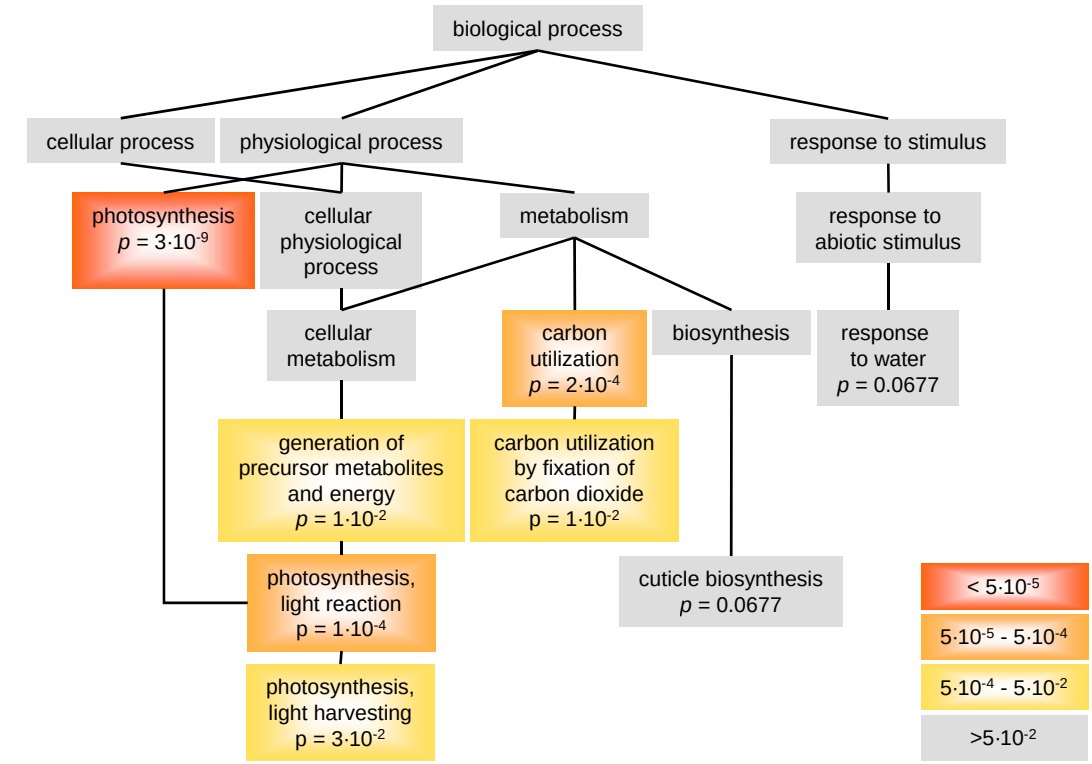

y = 0.93x + 0.29
R2 = 0.96
Microarray Log2 (35S:CcGA20ox1/wt)
RT-PCR Log2 (35S:CcGA20ox1/wt)
6
5
4
3
2
1
0
-1
-2
-3
-3
-2
-1
0
1
2
3
4
5
6

Figure 3

A


biological process
cellular process
physiological process
response to stimulus
photosynthesis
p = 3·10-9
cellular physiological process
metabolism
response to abiotic stimulus
cellular metabolism
carbon utilization
p = 2·10-4
biosynthesis
response to water
p = 0.0677
generation of precursor metabolites and energy
p = 1·10-2
carbon utilization by fixation of carbon dioxide
p = 1·10-2
photosynthesis, light reaction
p = 1·10-4
cuticle biosynthesis
p = 0.0677
photosynthesis, light harvesting
p = 3·10-2
< 5·10-5
5·10-5 - 5·10-4
5·10-4 - 5·10-2
>5·10-2

B

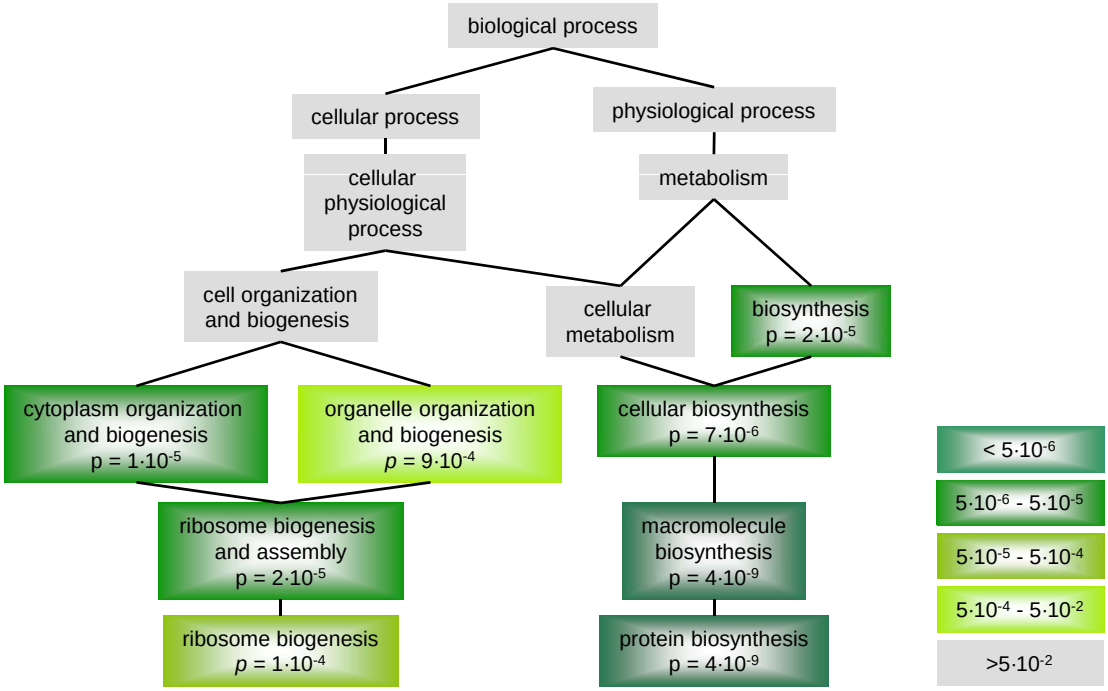
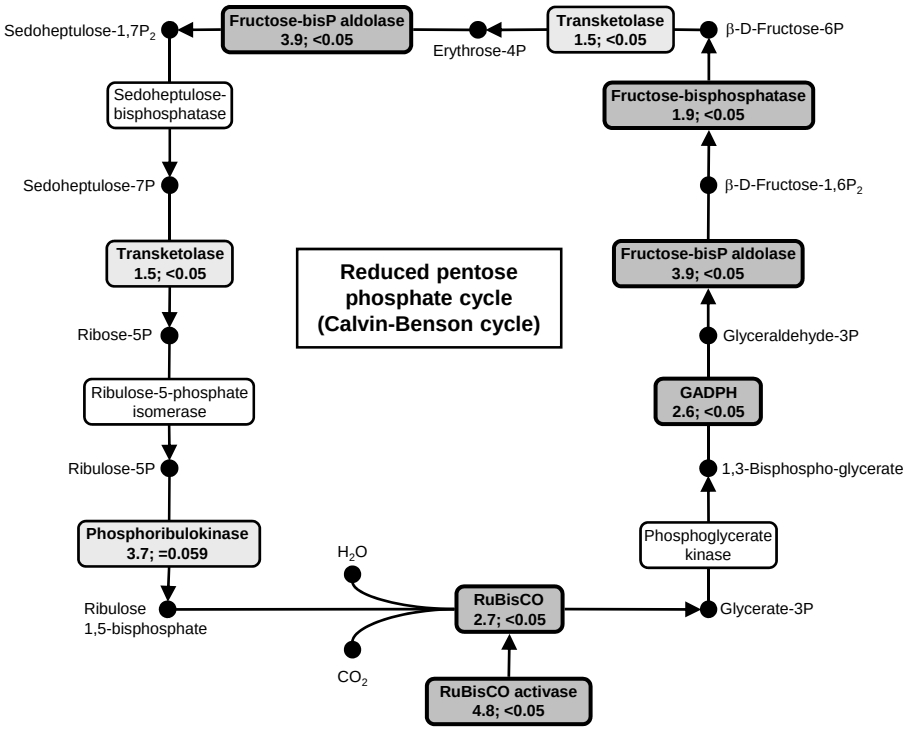

biological process
cellular process
physiological process
cellular physiological process
metabolism
cell organization and biogenesis
cellular metabolism
biosynthesis
p = 2·10-5
cytoplasm organization and biogenesis
p = 1·10-5
organelle organization and biogenesis
p = 9·10-4
cellular biosynthesis
p = 7·10-6
ribosome biogenesis and assembly
p = 2·10-5
macromolecule biosynthesis
p = 4·10-9
ribosome biogenesis
p = 1·10-4
protein biosynthesis
p = 4·10-9
< 5·10-6
5·10-6 - 5·10-5
5·10-5 - 5·10-4
5·10-4 - 5·10-2
>5·10-2

Figure 4


Sedoheptulose-1,7P2
Fructose-bisP aldolase
3.9; <0.05
Erythrose-4P
Transketolase
1.5; <0.05
β-D-Fructose-6P
Sedoheptulose-
bisphosphatase
Fructose-bisphosphatase
1.9; <0.05
Sedoheptulose-7P
β-D-Fructose-1,6P2
Transketolase
1.5; <0.05
Reduced pentose
phosphate cycle
(Calvin-Benson cycle)
Fructose-bisP aldolase
3.9; <0.05
Ribose-5P
Glyceraldehyde-3P
Ribulose-5-phosphate
isomerase
GADPH
2.6; <0.05
Ribulose-5P
1,3-Bisphospho-glycerate
Phosphoribulokinase
3.7; =0.059
Phosphoglycerate
kinase
H2O
Ribulose
1,5-bisphosphate
RuBisCO
2.7; <0.05
Glycerate-3P
CO2
RuBisCO activase
4.8; <0.05

Supplemental Figure 1

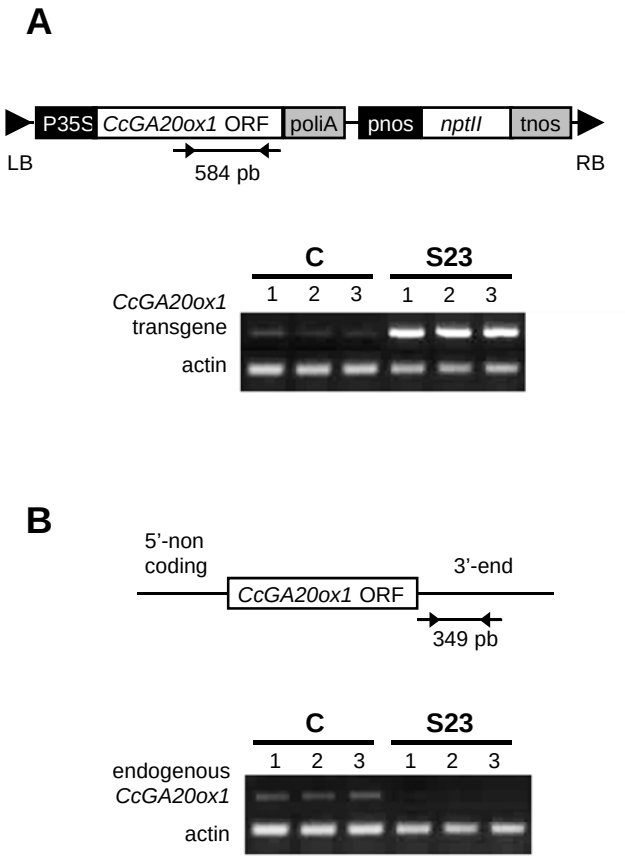


Table I. MIPS FunCat analysis of citrus differentially expressed genes in CcGA20ox plants

Note that there was 1122 gene annotations because 293 out of the 726 genes differentially expressed belonged to more than one functional category

FUNCTIONAL CATEGORY	Up-regulated		Down-regulated	
	No.	%	No.	%
01 METABOLISM	73	14,6	66	10,6
02 ENERGY	22	4,4	11	1,8
04 STORAGE PROTEIN	1	0,2		0,0
10 CELL CYCLE AND DNA PROCESSING	5	1,0	11	1,8
11 TRANSCRIPTION	10	2,0	9	1,4
12 PROTEIN SYNTHESIS	7	1,4	49	7,9
14 PROTEIN FATE (folding, modification, destination)	23	4,6	35	5,6
16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT (structural or catalytic)	60	12,0	99	15,9
18 REGULATION OF METABOLISM AND PROTEIN FUNCTION	5	1,0	7	1,1
20 CELLULAR TRANSPORT, TRANSPORT FACILITIES AND TRANSPORT ROUTES	32	6,4	32	5,1
30 CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM	8	1,6	9	1,4
32 CELL RESCUE, DEFENSE AND VIRULENCE	32	6,4	32	5,1
34 INTERACTION WITH THE ENVIRONMENT	35	7,0	24	3,9
36 SYSTEMIC INTERACTION WITH THE ENVIRONMENT	11	2,2	14	2,2
40 CELL FATE	3	0,6	6	1,0
41 DEVELOPMENT (Systemic)	8	1,6	13	2,1
42 BIOGENESIS OF CELLULAR COMPONENTS	8	1,6	14	2,2
43 CELL TYPE DIFFERENTIATION	2	0,4	3	0,5
47 ORGAN DIFFERENTIATION			3	0,5
70 SUBCELLULAR LOCALIZATION	111	22,2	142	22,8
77 ORGAN LOCALIZATION	3	0,6		
99 UNCLASSIFIED PROTEINS	40	8,0	44	7,1
Total number of gene annotations	499	100,0	623	100,0

Table II. *Effect of CcGA20ox1 overexpression on photosynthetic rate (PN, $\mu mol\ CO_2 m^{-2} s^{-1}$), stomatal conductance (Gs, mmol $m^{-2} s^{-1}$), transpiration (E, mmol $m^{-2} s^{-1}$) and water use efficiency (PN/E, $\mu mol\ CO_2$ /mmol H_2O)*

Measurements were performed in attached fully expanded young leaves of control and *CcGA20ox* plants. Each value represents means ± standar errors of 12 independent measurements. Values marked with a different letter are significantly different (P ≤0.05); letters before the comma correspond to values at each column, and letters after the comma to values at each file.

Genotype	Photosynthetically active radiation (PAR, $\mu mol\ CO_2 m^{-2} s^{-1}$)											
	600				800				1000			
	PN	Gs	E	PN/E	PN	Gs	E	PN/E	PN	Gs	E	PN/E
control	10,33 ± 0.84a,c	82,20 ± 4.33a,c	1,80 ± 0.08a,c	5,70 ± 0.29a,c	11,84 ± 1.04a,cd	70,60 ± 4.3a,c	1,57 ± 0.08a,c	7,44 ± 0.36a,d	14,45 ± 1.20a,d	80,1 ± 5.81a,c	1,65 ± 0.09a,c	8,67 ± 0.36a,e
CcGA20ox1	12,11 ± 1.53a,c	144,90 ± 20.27b,c	2,79 ± 0.28b	4,67 ± 0.68a,c	17,14 ± 1.06b,d	114,70 ± 13.07b,c	2,26 ± 0.18b,c	7,74 ± 0.36a,d	20,37 ± 1.24b,d	176,10 ± 21.94b,c	2,89 ± 0.25b,c	7,29 ± 0.43b,d

Table III. *Differentially expressed genes related to abiotic stress*

Description	GO terms	Citrus unigene	Fold-Change	Most similar Ath gene
Upregulated				
dehydrin family protein	response to water	aCL6Contig16	3,93	AT1G54410
		aCL6Contig7	3,41	AT1G54410
		aCL6Contig21	3,38	AT1G54410
late embryogenesis abundant like 5 (LEA5)	response to water deprivation	aCL9Contig8	2,96	AT4G02380
	response to oxidative stress	aCL6Contig22	2,61	AT4G02380
	response to reactive oxygen species	aCL9Contig19	2,40	AT4G02380
cysteine proteinase (RD21)	response to water deprivation	aCL23Contig3	2,30	AT1G47128
		aCL23Contig1	2,13	AT1G47128
delta 1-pyrroline-5-carboxylate synthetase (P5CS1)	response to water deprivation	aCL174Contig2	2,47	AT2G39800
	response to desiccation			
	response to salt stress			
	hyperosmotic salinity response			
	response to abscisic acid stimulus			
9-cis-epoxycarotenoid dioxygenase (CCD1)	response to water deprivation	aCL920Contig2	2,16	AT3G63520
zeaxanthin epoxidase (ABA1)	response to water deprivation	aCL1551Contig1	2,18	AT5G67030
	response to osmotic stress	aCL3421Contig1	1,79	AT5G67030
cold acclimation protein COR413-TM1	cellular response to water deprivation	aCL5208Contig1	2,04	AT1G29395
	response to abscisic acid stimulus			
glutathione S-transferase (GST8)	cellular response to water deprivation	aCL87Contig1	1,97	AT1G78380
	response to oxidative stress			
cysteine proteinase (RD19)	response to water deprivation	aCL96Contig1	1,68	AT4G39090
	response to desiccation			
	response to osmotic stress			
	response to salt stress			
dehydration-responsive protein (RD22)	response to desiccation	aCL172Contig1	5,84	AT5G25610
	response to salt stress	aCL172Contig2	5,81	AT5G25610
	response to abscisic acid stimulus			
zinc finger (C3HC4-type RING finger)	response to osmotic stress	aCL198Contig1	2,18	AT2G04240
	response to salt stress			
synaptobrevin family protein (VAMP714)	response to salt stress	aCL1312Contig1	1,65	AT5G22360
isoflavone reductase	response to oxidative stress	aCL4218Contig1	2,90	AT1G75280
peroxidase 42 (PER42)	response to oxidative stress	aCL36Contig3	1,73	AT4G21960
		aCL36Contig2	1,63	AT4G21960
chalcone synthase (CHS)	response to oxidative stress	aCL1023Contig1	2,17	AT5G13930
Downregulated				
NAC transcription factor (RD26)	response to water deprivation	aCL35Contig3	-1,69	AT4G27410
	response to abscisic acid stimulus			
lipoxygenase (LOX2)	response to water deprivation	aCL241Contig1	-3,83	AT3G45140
peroxidase 3 (RCI3)	response to desiccation	aCL622Contig2	-3,36	AT1G05260
	hyperosmotic salinity response			
alcohol dehydogenase (ADH1)	response to osmotic stress	aCL2951Contig1	-2,11	AT1G77120
myb-related transcription factor (CCA1)	response to salt stress	aCL2656Contig1	-1,82	AT1G01060
	response to abscisic acid stimulus			
myb family transcription factor (MYB78)	response to salt stress	aCL7866Contig1	-2,33	AT5G49620
	response to abscisic acid stimulus			
tonoplast intrinsic protein (TIP)	response to salt stress	aCL62Contig1	-2,04	AT2G36830
L-ascorbate peroxidase 3 (APX3)	response to oxidative stress	aCL7975Contig1	-1,74	AT4G35000
peroxidase	response to oxidative stress	aCL622Contig1	-2,16	AT5G15180
chalcone synthase (CHS)	response to oxidative stress	aCL27Contig2	-1,92	AT5G13930
calreticulin 1 (CRT1)	response to oxidative stress	aCL1164Contig1	-2,34	AT1G56340

Table IV. *Differentially expressed genes encoding transcription factors*

	Upregulated			Downregulated		
	Citrus unigene	Fold-Change	Most similar Ath gene	Citrus unigene	Fold-Change	Most similar Ath gene
transcription factor IIB (TFIIB) family protein	aCL656Contig3	2,41	AT4G36650			
high mobility group B protein (HMGB4)				aCL138Contig4	-1,60	AT2G17560
CDC2-related kinase subfamily (AFC1)				aCL3136Contig1	-6,77	AT3G53570
myb-related transcription factor (CCA1)				aCL2656Contig1	-1,82	AT1G01060
MYB Transcription Factor Family						
myb family transcription factor (MYB52)	aCL5017Contig1	1,94	AT1G17950			
myb family transcription factor (MYB121)				aCL2843Contig1	-3,13	AT3G30210
myb family transcription factor (MYB78)				aCL7866Contig1	-2,33	AT5G49620
CCAAT-HAP5 Transcription Factor Family						
heme activated protein (HAP5c)	aCL665Contig2	1,92	AT1G08970			
C2C2-YABBY Transcription Factor Family						
plant-specific transcription factor YABBY	aCL4648Contig1	1,78	AT2G26580			
Homeobox Transcription Factor Family						
BEL1-like homeodomain 1 (BLH1)	aCL157Contig1	2,81	AT2G35940			
BEL1-like homeodomain 1 (BLH1)	aCL1577Contig1	1,94	AT2G35940			
class II knotted1-like homeobox (KNAT3)	aCL1472Contig1	1,94	AT5G25220			
bHLH Transcription Factor Family						
basic helix-loop-helix (bHLH) family protein	aCL9380Contig1	1,68	AT3G07340			
WRKY Transcription Factor Family						
WRKY family transcription factor (WRKY31)	aCL1201Contig1	1,64	AT4G22070			
ABI3VP1 Transcription Factor Family						
transcriptional factor B3 family protein (VRN1)	aCL7325Contig1	1,70	AT3G18990			
bZIP Transcription Factor Family						
bZIP family transcription factor				aCL6889Contig1	-5,32	AT1G08320
C2H2 Transcription Factor Family						
zinc finger family protein (SUF4)				aCL335Contig1	-1,62	AT1G30970
NAC Transcription Factor Family						
NAC transcription factor (RD26)				aCL35Contig3	-1,69	AT4G27410
Response Regulator Gene Family						
pseudo-response regulator 5 (APRR5)	aCL5406Contig1	1,60	AT5G24470			