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Tetraploid Rangpur lime rootstock increases drought tolerance via enhanced constitutive root abscisic acid production

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ABSTRACT

Whole-genome duplication, or polyploidy, is common in many plant species and often leads to better adaptation to adverse environmental condition. However, little is known about the physiological and molecular determinants underlying adaptation. We examined the drought tolerance in diploid (2x) and autotetraploid (4x) clones of Rangpur lime (*Citrus limonia*) rootstocks grafted with 2x Valencia Delta sweet orange (*Citrus sinensis*) scions, named V/2xRL and V/4xRL, respectively. Physiological experiments to study root–shoot communication associated with gene expression studies in roots and leaves were performed. V/4xRL was much more tolerant to water deficit than V/2xRL. Gene expression analysis in leaves and roots showed that more genes related to the response to water stress were differentially expressed in V/2xRL than in V/4xRL. Prior to the stress, when comparing V/4xRL to V/2xRL, V/4xRL leaves had lower stomatal conductance and greater abscisic acid (ABA) content. In roots, ABA content was higher in V/4xRL and was associated to a greater expression of drought responsive genes, including *CsNCED1*, a pivotal regulatory gene of ABA biosynthesis. We conclude that tetraploidy modifies the expression of genes in Rangpur lime citrus roots to regulate long-distance ABA signalling and adaptation to stress.

Key-words: citrus; ABA; adaptation; polyploidy; water deficit.

INTRODUCTION

Polyploidy is a frequent occurrence in the plant kingdom (Masterson 1994) and is a major force of plant evolution (Soltis & Soltis 2009; Chen 2010). Polyploids may be allotetraploids or autotetraploids, resulting either from sexual reproduction via 2n gametes or somatic chromosome doubling, respectively. Thus, allotetraploids inherit subgenomes from two different parents after interspecific hybridization. In citrus, it is important to note that somatic hybridization is a major source of allotetraploids (Dambier *et al.* 2011; Grosser & Gmitter 2011). Autotetraploids, such as most tetraploid citrus genotypes, arise from somatic chromosome doubling and from intraspecific hybridization or self-fertilization through 2n gametes. The subgenomes of these autotetraploids are considered to be identical (Aleza *et al.* 2011).

Numerous studies focusing on the genetic and epigenetic changes associated with polyploidization have been carried out on newly created allopolyploid materials (Lukens *et al.* 2006; Rapp, Udall & Wendel 2009). Ni *et al.* (2009) proposed that hybrids and allopolyploids have improved control of circadian-mediated physiological and metabolic pathways, and that such changes increase growth vigour and biomass. Those changes in gene expression are potentially advantageous in allopolyploids because an increase in heterozygosity may be at the origin of subfunctionalization (Adams *et al.* 2003). In allotetraploids, changes in gene expression are thought to be the result of genome hybridization rather than changes in genome ploidy (Auger, Peters & Birchler 2005; Chen 2010). On the other hand, few studies have sought to identify changes in the genome expression pattern of autotetraploids (Stupar *et al.* 2007; Riddle *et al.* 2010; Yu *et al.* 2010). In contrast to allotetraploids, alteration in the expression of any allele in autopolyploids may lead to a change in phenotype, because no potential complementation or advantageous subfunctionalization could occur. Interestingly, investigations of autopolyploid series in potato, maize and citrus showed that gene expression changes were very limited among plants in the series (Stupar *et al.* 2007; Riddle *et al.* 2010; Allario *et al.* 2011; Li *et al.* 2012). These results suggest that large phenotypic differences probably correlate with subtle changes in gene expression, rather than with extensive transcription reformatting. Recent work in autotetraploid *Arabidopsis thaliana* showed that the alteration in transcriptome response does not depend on the chromosome number per se but on the origin of the chromosome (Yu *et al.* 2010). Studies of genome expression in polyploid plants subjected to stress showed that genes duplicated by polyploidy in ancestral polyploids are preserved in their genomes because their expression has been partitioned in response to environmental stress (Liu & Adams 2007). Ramsey (2011) recently investigated the adaptation of polyploid populations to novel environments and analysed the evolutionary consequences

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of exposure to these environments. He observed that hexaploid wild yarrow (*Achillea borealis*) and, to a lesser extent, the neo-hexaploid had a fitness advantage over the tetraploids. He concluded that not only polyploidization per se, but also genetic evolution after the polyploidization event, confers improved adaptation to the environment.

Grafting provides an opportunity to investigate the effect of polyploidy on roots versus polyploidy in the entire plant, and there has been continuous strong interest in citrus tetraploid rootstocks in the past decades (Barrett & Hutchinson 1982; Grosser & Chandler 2003; Aleza *et al*. 2011). Tetraploid citrus have lower rates of whole plant transpiration, which is associated with lower growth rates, than their respective 2x parents (Syvertsen, Lee & Grosser 2000; Allario *et al*. 2011). The recent work we performed demonstrated that the use of 4x rootstocks dramatically changes both tree physiology and fruit yield, without promoting large changes in fruit quality criteria (Hussain *et al*. 2012). Furthermore, based on seedling growth and chloride accumulation in leaves exposed to salinity stress, 4x citrus seedlings were shown to be more stress tolerant than their respective 2x parents (Saleh *et al*. 2008).

In this study, we investigated the water deficit tolerance of 2x and 4x clones of Rangpur lime (*Citrus limonia*) rootstocks grafted with 2x Valencia Delta sweet orange (*Citrus sinensis*) scions. The use of a 2x variety allowed us to work with a scion that did not present any phenotypic differences, such as the ones observed between leaves of 2x and 4x citrus seedlings (Allario *et al*. 2011). It has been possible to determine whether the roots of 4x genotypes are involved in the adaptation to drought by monitoring a specific physiological aspect, such as a change in abscisic acid (ABA) signalling. Finally, the rootstock/variety associations on which we report correspond to combinations that are commonly planted in orchards, underlining the economic importance of this research.

MATERIALS AND METHODS

Plant material

Seeds from diploid (2x) (IVIA-334) and autotetraploid (4x) (IVIA-516) of Rangpur lime (*C. limonia*, Osbeck) were collected from trees of the IVIA germplasm collection in Spain. Originally, the spontaneous autotetraploid genotype (4x) was selected at IVIA among a population of seedlings from the diploid plants. Seeds were planted in substrate (sand, turf and peat, 1:1:1) in a greenhouse for 4 months. The ploidy status of 2x and 4x seedlings was checked and confirmed by flow cytometry (Partec I) according to Froelicher *et al*. (2007). The genetic composition of 4x seedlings compared with 2x was analysed using 10 simple sequence repeats (SSRs) according to Allario *et al*. (2011).

Thirty 2x and 4x 1-year-old seedlings were grafted with 2x Valencia Delta sweet orange (*C. sinensis*, IVIA 363). 5 T-budding was performed 20 cm above the ground. Trees were grown in the greenhouse for 2 years with day/night temperatures of 20–35 °C/18–20 °C, respectively, and relative humidity varying between 40 and 85%. As V/4xRL trees grew slower than V/2xRL, trees were pruned to equalize tree size. Trees were then transplanted in new 4 L pots containing fresh commercial soil, and regular fertilization was applied according to Allario *et al*. (2011). Four months after pruning, 24 V/2xRL and 24 V/4xRL uniform trees presenting therefore a similar number of leaves for V/2xRL and V/4xRL trees were selected to investigate water deficit tolerance.

Water deficit application

Trees were regularly watered at field capacity for 2 weeks prior to applying the water deficit. Each pot of the first set of trees was enclosed in a plastic bag. Water requirements to maintain control trees at field capacity were estimated. Six V/2xRL and six V/4xRL were assigned as control trees. The water deficit was applied by ceasing watering of six tree combinations of V/2xRL and six V/4xRL for 24 d after bagging the pots. For a second set of 12 V/2xRL and 12 V/4xRL trees, six trees of each combination were assigned as control trees, and six trees of each combination were subjected to a water deficit applied without bagging the pots by stopping the irrigation for 11 d in order to induce a faster stress response.

Leaf physiological parameters, ABA content

Mature leaves were selected and labelled to avoid leaf anatomy changes and significant developmental adaptation along the stress experiment. Leaf relative water content (RWC) was measured in control condition according to Barr & Weatherly (1962). Leaf stomatal conductance (g_s) was also 6 monitored using a leaf porometer (SC-1; Decagon Device, 7 Pullman, WA, USA). The quantum yield of photosystem II electron transport (F_m/F_v), which represents the photosystem II activity, was checked using a leaf fluorometer (Fluorpen FP 100; Photon Systems Instruments, ••, ••) (Percival 2005). 8 Each measurement (g_s and F_m/F_v) was taken between 0900 and 1100 h on 3 mature leaves per plant and 6 plants per genotype (18 replicates). Slides for the analysis of stomatal size and the number of stomata per unit of leaf surface area (stomatal density) were prepared according to Morillon & Chrispeels (2001). Two segments of the same mature leaf were stuck to the same microscope slide. Three different slides from three different leaf trees were done for each tree combination. A total of 60 stomata were measured. Pots were regularly weighed during the experiment to measure the water consumption. Six independent biological root and leaf sample replicates were harvested from each tree combination (from 0900 to 1200 h of the day) to prevent any potential interference of circadian rhythms. Six independent biological samples were harvested and frozen in liquid nitrogen on the last day of the experiment. Three root and leaf samples, each one corresponding to a mix of two previously harvested samples, were used for ABA assays according to Xiong *et al*. (2001). ABA concentration was determined using a Phytodetek ABA Immunoassay Kit, ••, ••.

RNA extraction and labelling

For microarray analyses, 4 independent biological replicates per tree combination and condition were randomly selected among leaf samples. Total RNA purification and a quality assessment were performed as previously described by Brumós *et al*. (2009). Total RNA was retrotranscribed to cDNA and labelled with the Cy3 and Cy5 fluorophores, following the indirect method, as previously described (Forment *et al*. 2005). Dye incorporation and cDNA recovery were determined by ultraviolet (UV) absorption spectrophotometry using the NanoDrop spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific, ••, DE, USA).

Microarray hybridization and analysis

The *Citrus* genome-wide cDNA microarray was used, which includes 21 081 putative unigenes described by Martinez-Godoy *et al*. (2008). Two independent microarray hybridization experiments with leaves of the 2x Valencia Delta orange alternatively grafted onto 2x or 4xRL rootstocks were respectively performed. For each experiment, the Cy-labelled cDNA of control leaves was hybridized with labelled cDNA from water-stressed samples. To avoid Cy3 and CY5 dye-related artefacts, control and water-stressed cDNA samples were dye swapped and used to hybridize four slides corresponding to different combinations of the four biological replicates obtained from every treatment. The microarray hybridizations were performed according to Allario *et al*. (2011). Cy5-labelled cDNA from individual samples was mixed with equal amount of Cy3-labelled reference sample (40–60 pmol of each dye) in 55 μL hybridization solution containing 3xSSC, 0.1% SDS and 0.1 mg mL^{-1} salmon sperm DNA. The hybridization mix was denatured at 95 °C for 60 s and applied to a microarray slide previously pre-hybridized. Slide pre-hybridization consisted of 60 min incubation at 50 °C in 3xSSC, 0.1% SDS and 0.1 mg mL^{-1} BSA, washing twice with SSC (0.1x) and once with distilled water, followed by drying through 5 min centrifugation at 1000 *g*. The slide was placed in a hybridization chamber (Olympus, Tokyo, Japan) and incubated overnight (14–16 h) in a bath at 50 °C. After hybridization, slides were washed by stepwise incubations in 2xSSC + 0.1% SDS buffer at 42 °C for 5 min, 0.1x SSC + 0.1% SDS buffer at 28 °C for 5 min (twice), 0.1xSSC at 28 °C for 1 min (five times), and finally in 0.01xSSC buffer at 28 °C for some seconds. Drying was achieved by centrifugation at 1000 *g* for 5 min.

A GenePix 4000B microarray scanner (Axon Instruments, Inc., Union City, CA, USA) and the GenePix Pro 4.1 acquisition software were used to scan the chips at 5–10 μm resolution. Photomultiplier gains for the two channels were adjusted so that the ratio of total intensities was approximately 1 and the percentage of saturated spots was about 1%. High-resolution tiff images were generated and used for quantification of gene expression data. Spot positions were identified on the colour images and quality flags were assigned to individual spots both automatically and manually. Only spots with background-subtracted foreground intensity greater than 2 in at least one channel were used, and only microarrays with optimal hybridization data were pre-processed and normalized for further analyses. Raw data were imported into the R-computing environment for pre-processing, visualization and statistical analysis. To identify probes showing significant differential gene expression between samples, the linear models for microarrays (LIMMA; Smyth 2005) software package was used. Pre-processing and normalization of two-colour microarray data including signal intensity, background correction, uniformity of the expression ratio over the chip surface (within-array normalization), and normality of *M*-value distributions were evaluated according to Smyth and Speed (2003). *M*-value was defined as the logarithm in base-2 of water deficit versus control expression ratio. Reproducibility between replicates that were assessed indicated that the experimental system provided consistent signals in spots corresponding to the same gene and acceptable low variability between biological replicates (not shown). *P*-values associated to the statistical analysis of differential expression obtained from LIMMA analysis are corrected for multiple comparisons using the B&H false discovery rate (FDR) procedure (Benjamini & Hochberg 1995; Reiner, Yekutieli & Benjamini 2003). Differences in gene expression were considered to be significant when the *M*-value was ≥/0.7/ and the FDR-adjusted *P*-value was smaller than 0.05

Functional analysis

Gene ontology (GO; Ashburner *et al*. 2000) term annotation of array features and function-based analysis of microarray results were carried out with Blast2GO (Conesa *et al*. 2005). The GO terms for each of the three GO main categories (biological process, molecular function and cellular component) were obtained from sequence similarity analysis using the application default annotation parameters. GO annotations were completed by locally running homology searches according to Quevillon *et al*. (2005) and applying the second layer GO augmentation strategy (Myhre *et al*. 2006). GO term enrichment analysis of significantly differentially expressed genes was also performed (Bluthgen, Kielbasa & Herzel 2005). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO; Edgar, Domrachev & Lash 2002) and are accessible through GEO Series accession number GSE41311.

Real-time RT-PCR

Genes that were selected from microarray analysis for (q)RT-PCR investigations were searched for among the sequences in the non-redundant databases using the BLASTN and TBLASTX algorithms and in the citrus EST database (CFGP database, Citrus Functional Genomic project, http://bioinfo.ibmcp.upv.es/genomics/cfgpDB/bioinfo.ibmcp.upv.es). Primers used for quantitative real-time RT-PCR (Table 1) were defined using the software Oligo Explorer (Gene Link_, http://www.genelink.com/tools/gl-oe.asp) to select primer pairs with the lowest possible

Table 1. Citrus gene accession number and function and primers used by the selected genes to be confirmed by RT-qPCR results of expression obtained through microarray analysis

Gene name	Accession No.	Function of the gene or the encoded protein	Primer sequence (5′->3′)
Repressed genes			
Carbonic anhydrase (*CA*)	C01015D11	Carbonic anhydrases form a family of enzymes that catalyse the rapid conversion of carbon dioxide to bicarbonate and protons and favour photosynthesis	F: CACCAGCTCCTATCATCAAC R: GCAACAGGTTTCAAGTCTTC
Chloroplast oxygen-evolving enhancer protein (*OEE*)	C31604E09	Oxygen-evolving enhancer proteins are auxiliary components of the photosystem II manganese cluster	F: CTCAGGTTCCTTTGTTCAAG R: TGGCTCATCAGAGTTCAAC
Phosphoenolpyruvate carboxylase 2 (*PEPC*)	C16013C03	Phosphoenolpyruvate carboxylases are key enzymes of photosynthesis by catalysing the addition of CO_2 to phosphoenolpyruvate to form the four-carbon compound oxaloacetate	F: CCAAGCCTACACTCTGAAG R: TAGGGTTAAGCCTCACAAG
Photosystem II reaction centre W protein chloroplast precursor (*psbW*)	C31604G05	Subunit of the photosystem II	F: CTGTTGGGTGTTTTTGGTC R: TGGGTTTGGCTTTAGACTTC
CuZn superoxide dismutase (*SOD*)	C31504C12	CuZn superoxide dismutases are mostly involved ROS detoxication	F: GGAACTGTTTCCTTTAGCG R: TGCCTATGTTTCCGTAAGTG
Dehydroascorbate reductase (*DHAR*)	C31402E11	Dehydroascorbate reductase allows the maintaining of an appropriate level of ascorbate in plant cells that protect against oxidant injury	F: AGCCCAGAAGGGAAAGTACC R: AGGCAAATTCAGGAGGATTG
Glutathione S-transferase GST 22 (*GST*)	C07010G07	Glutathione S-transferase catalyses the conjugation of reduced glutathione and favours detoxication of endogenous compounds such as peroxidized lipids	F: AATCGCGAGCTATCATAAGG R: CTACTTCCAGCCATTGTTCC
Putative fatty acid elongase (*FAE*)	C32009D10	Fatty acid elongase would be involved in bilayer making as well as in the wax of the cuticle	F: GGGGCTTGAAGAATACAGG R: AATGCCTCAGCTAAAGAAGG
Overexpressed genes			
Chlorophyllide A oxygenase chloroplast precursor (*CAO*)	C31601G07	Chlorophyllide A oxygenase chloroplast precursors are enzymes involved in chlorophyll *b* biosynthesis	F: TACCTATGGAGGCACTTTG R: CACCCCTAGTTTGTCGTAAC
Alternative oxidase mitochondrial precursor (*AOX*)	KN0AAP7YK07	Alternative oxidases are enzymes that provide an alternative route for electrons passing through the electron transport chain to reduce oxygen and enhance an organism's ability to resist stresses through reducing the level of oxidative stress	F: GCGTAAGTTCCAGCATAGTG R: CCTCCAAGTAGCCAACAAC
Cinnamoyl-CoA reductase-like protein (*CCR*)	C34205C03	Cinnamoyl-CoA reductases are enzymes that catalyse the first step of biosynthesis of monoterpenes of lignin	F: CCTTGCAAAGACACTATCTG R: GATTGAGGGTTCTGTTGAG
Cuticle protein (*WAX2*)	C02002B06	Compound of the cuticle on the leaf epidermis that allows to limit leaf water loss and limit irradiation caused by the sun	F: CTCGATGGAACACAAAAGG R: AGTGGTAATGGGTGAAAAGG
Putative *HVA22* gene	C31106H02	*HVA22* gene was shown to be induced by environmental stresses, such as dehydration, salinity, and extreme temperatures, and by a plant stress hormone, abscisic acid	F: TGGCAAGGGCAAGAACAAG R: ACAAGCCAACGGAGAATCG
Dehydrin (*DH*)	C31207C07	Dehydrins are thermostable hydrophilic proteins that play a major role of osmoprotectant and stabilize proteins in stress condition	F: GCCACCGAGTTTGAGAAAG R: GTGGATCGGTGAAGTTTGTC
Delta 1-pyrroline-5-carboxylate synthetase (*P5CS*)	C34107H03	Delta 1-pyrroline-5-carboxylate synthetase allows conversion of glutamate to Delta 1-pyrroline-5-carboxylate to proline that is involved in osmotic stress tolerance	F: CTAGGAAAGCACCATACGAG R: GAGGCCCTCTACATCACTC
Early-responsive to dehydration protein (*ERD4*)	C01019H06	Early-responsive to dehydration proteins were shown to be synthesized in water deficit condition and would favour protein stabilization	F: CGCTGCCCTGCTACTGTAC R: CCATGCTAGGGGTTTCCTTC
Galactinol synthase (*GoLS*)	C31402D06	Galactinol synthase catalyses the first step of the biosynthesis of raffinose family oligosaccharides, which are osmoprotectant against water deficit	F: CCATGGCCTATTATGTCATC R: CATCAGGCAAGTCAAACAG
Group I late embryogenesis abundant protein (*LEA*)	C34209G11	Group I late embryogenesis abundant proteins were shown to synthesize in water deficit condition and would favour protein stabilization	F: GCGACGGAGAAGAAAGAGG R: CACCACCCCTTCAATCACC
Raffinose synthase (*RS*)	C05076C10	Raffinose synthase enzyme allows the biosynthesis of the raffinose known to be an osmoprotectant when plants are subjected to water deficit	F: TCGACGTTATCCATTTGCTG R: CACCATTGCCCTTGAAGTG

The rate of expression corresponds to the gene expression in stress condition over control condition from Valencia leaf samplings of 2xRL/V and 4xRL/V (Supporting Information Fig. S2). Annealing temperature was 55°C.
ROS, reactive oxygen species.

number of potential primer dimers and primer hairpins. Quantitative real-time RT-PCR was performed according to Colmenero-Flores *et al*. (2007). For each investigated gene, three biological replicates were analysed.

Statistical analysis

Data are expressed as the mean value ± SE. SIGMASTAT from SPSS (Chicago; http://www.spss.com_software_science) was used to analyse the data. Analysis of variance (ANOVA) and the Student's *t*-test were used to detect differences between the genotypes and the growing conditions at the usual probability level lower than 0.05. Data normalization and transformation were performed when needed.

RESULTS

Tetraploid acquisition and plant genetic constitution

Using flow cytometry, no 2x seedling was found among seedlings obtained from seeds of the 4x mother tree, and no 4x seedling was found among seedlings obtained from seeds of the 2x mother tree. Analysis of the genetic constitution of 2x and 4x genotypes using SSR molecular markers showed that marker profiles were identical between 2x and 4x genotypes (data not shown), proving that the 4x genotype is an autotetraploid originating from a perfect duplication of a diploid genome.

Growing in control conditions, shoot–water relations of 2x scion are modified when grafted on the 4x rootstock

To investigate whether 4xRL rootstock can influence the leaf water content of the scion, different physiological parameters of leaves including the water balance of V/2xRL and V/4xRL trees were investigated. Leaf surface area and fresh weight were not significantly affected by the ploidy level of the rootstocks. Furthermore, the ploidy status of the rootstock did not alter the RWC of the scion leaves (Table 2). Additionally, mature leaf stomatal density and size were not significantly different between V/2xRL and V/4xRL (Table 2). Interestingly, under well-watered conditions, the leaves of V/4xRL trees had a lower stomatal conductance (g_s) than those of V/2xRL, with values of 94.0 and 126.7 mmol m^{-2} s^{-1}, respectively (Fig. 1a).

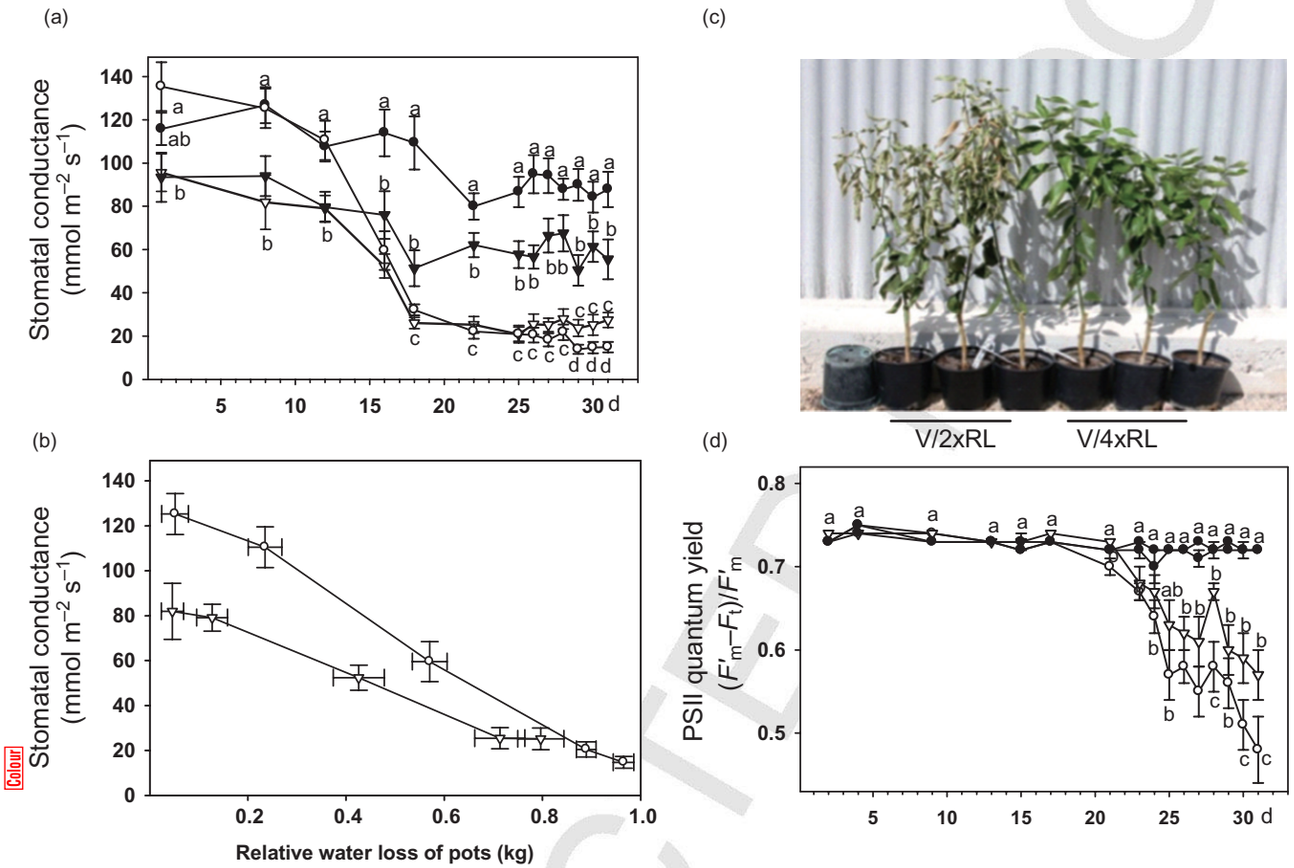
4x rootstock conferred improved tolerance to water deficit

V/2xRL and V/4xRL trees in bagged pots were subjected to 24 d of water deficit subsequent to 7 d of control treatment, and after 31 d the experiment was terminated and the roots and leaves were harvested for ABA analysis; to measure water usage, the pots were weighed. As the pots were bagged, water loss occurred only via the transpiration stream, and pot weight decreased slowly once water was being withheld. Wilting was not observed in either V/2xRL or V/4xRL trees grown in bagged pots even at the end of the experiment (31 d). Even though evolutions in the g_s values of the leaves of the control V/2xRL and V/4xRL trees were observed due to changes of the greenhouse conditions along the experiment, V/4xRL leaf g_s values remained all the time significantly lower (dark symbols, Fig. 1a). For the stressed trees (open symbols), there was no change until the 16th day of the experiment (9 d of water deficit), and g_s reached a plateau on the 18th day. By the 31st day, g_s continued to decrease to a value below 20 mmol m^{-2} s^{-1} for V/2xRL but not for V/4xRL (Fig. 1a). Plotting stomatal conductance versus relative water loss of the pots clearly indicated that V/4xRL trees presented lower g_s values than V/2xRL at initial and more limited water consumption at the end of the experiment (Fig. 1b). When pots were bagged, V/2xRL exhibited a faster decrease in pot weight than V/4xRL, corresponding at the end of the experiment to the higher value of g_s in V/4xRL than in V/2xRL trees. When pots were not bagged, trees experienced a much pronounced and faster water deficit. After an 11 d water deficit, V/2xRL genotypes were clearly more wilted than V/4xRL (Fig. 1c). The leaf quantum yield of photosystem II (Φ_{PSII}) of these trees was also measured. For stressed trees, Φ_{PSII} dropped after the decrease in g_s, and V/2xRL trees were more affected by the stress (Fig. 1d). As stomatal conductance is known to be related to ABA content, we measured ABA levels in leaves and roots. In control conditions, V/4xRL leaves contained twice as much ABA than V/2xRL leaves (Fig. 2a) and fivefold more ABA in the roots (Fig. 2b). Under stress conditions, at the end of the experiment, the ABA content was similar in the leaves and roots of V/2xRL and V/4xRL trees.

Table 2. Surface, thickness and relative water content (RWC) of the Valencia scion leaves, stomatal size, and density in mature leaves of V/2xRL and V/4xRL trees grown in control condition

	V/2xRL (mean ± SE)	V/4xRL (mean ± SE)
Leaf surface (cm^2)	36.02±1.6a	33.96 ± 1.9a
Leaf thickness (mm^2)	0.241 ± 7a	0.254 ± 6a
Relative water content of the scion leaves (%)	83.5a	84.3a
Stomata size (length × width, μm^{-2})	290±25a	310±19a
Stomata density (stomates /mm^2)	360±27a	385±23a

All values are averages of 10–60 replicates ± SE. Data with the same letters are not statistically different.



Colour

Figure 1. The effect of a water deficit on V/2xRL and V/4xRL. (a) Leaf stomatal conductance (g_s) of V/2xRL and V/4xRL trees grown in bagged pots in control conditions and subjected to a water deficit for 24 d ($n = 18$). (b) Stomatal conductance was plotted versus the relative water loss of the pots ($n = 6$). For each genotype, measurements were performed the same day (day 8, day 12, day 16, day 26 and day 30). Only day 8 and day 12 did not present a significant difference of relative water loss between V/2xRL and V/4xRL trees. (c) Photograph of V/2xRL and V/4xRL trees grown in non-bagged pots after 11 d of water deficit. (d) Quantum yield of photosystem II (PSII) [$(F'_m\text{-}F_t)/F'_m$] measured in the same genotypes ($n = 18$). (●: V/2xRL, control condition; ○: V/2xRL, stress condition; ▼: V/4xRL, control condition; ▽: V/4xRL, stress condition). Data with a different letter are statistically different. Vertical bars indicate the mean value ± SE.

In control conditions, 4x rootstocks constitutively overexpressed a set of genes involved in water deficit tolerance

Global gene expression changes induced by water deficit were monitored in the leaves of V/2xRL and V/4xRL bagged trees by microarray hybridization and RT-qPCR. We used samples harvested on the last day of the experiment (time-based comparison) since at this time, V/2xRL trees exhibited a much stronger stress phenotype for water loss and stomatal conductance than V/4xRL trees (Fig. 1a–c). Only information from microarrays with optimal hybridization data was pre-processed and normalized for further analyses (see Materials and Methods section). V/2xRL trees had a greater transcriptional response to water deficit than V/4xRL, because 896 and 342 genes were differentially expressed in the leaves of V/2xRL and V/4xRL, respectively (Fig. 3a). The ratio of induced/repressed genes was 0.48/0.52 and 0.43/0.57 for V/2xRL and V/4xRL trees, respectively (Fig. 3b; see Supporting Information Tables S1 and S2 for detailed lists of differentially expressed genes). Out of the 342 genes differentially expressed in V/4xRL, 230 (67%) were also differentially expressed in V/2xRL, and the subset of common genes exhibited a similar expression pattern (Supporting Information Fig. S1). Functional categories significantly enriched in the groups of water deficit-induced and -repressed genes were additionally identified through Fisher's exact test with multiple testing correction according to Conesa *et al.* (2005). Significantly enriched functional categories induced by water deficit were similar in both V/2xRL and V/4xRL trees (Supporting Information Table S3). The most abundant categories in the group of induced genes, common to V/2xRL and V/4xRL trees, were related to abiotic stress responses, including ABA and water deprivation responses (Fig. 3c and Supporting Information Table S3). The most significant difference observed in the group of induced genes was related to 'polysaccharide catabolic processes' and related categories, which were significantly enriched in V/2xRL plants but not in V/4xRL plants (Supporting Information Table S3). A different scenario was found in the group of genes repressed

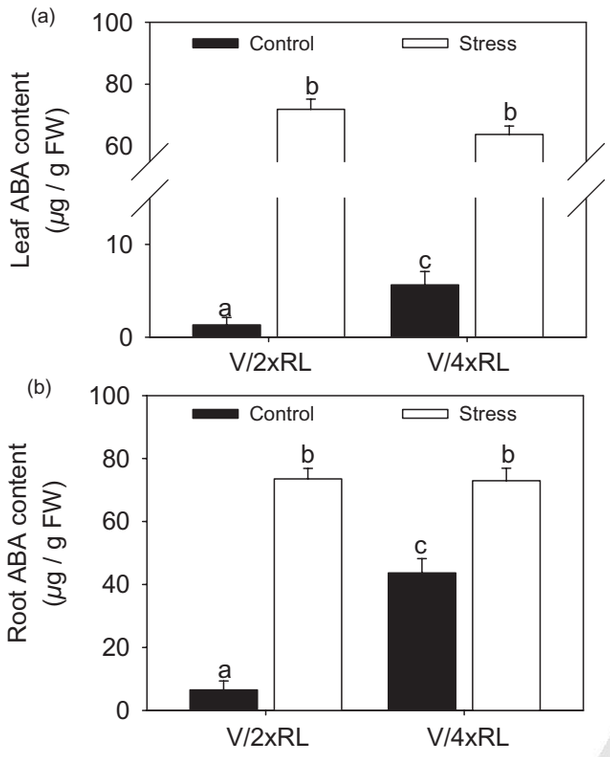


Figure 2. Abscisic acid (ABA) content of V/2xRL and V/4xRL organs of trees grown in bagged pots in control conditions and after 24 d of stress. (a) Leaf ABA content of V/2xRL and V/4xRL trees. (b) Root ABA content of V/2xRL and V/4xRL trees. For each graph, data with different letters are statistically different. Vertical bars indicate the mean value ± SE ($n = 3$).

by water deficit because they revealed significant differences between the response to water deficit of V/2xRL and V/4xRL trees (Supporting Information Table S4). Genes related to photosynthesis, carbohydrate metabolism, carbon fixation, jasmonic acid (JA) biosynthesis, response to JA stimulus, response to salicylic acid (SA) stimulus, defence response, JA and ethylene-dependent systemic resistance, and toxin catabolism were significantly more repressed in Valencia grafted onto 2x rootstocks than 4x rootstocks (Supporting Information Tables S4 and S5). Other functional categories related to starch biosynthesis and protease inhibitor activities were repressed to a similar extent in both systems (Supporting Information Table S4).

In order to validate the microarray data, transcript abundance of genes associated with photosynthesis, detoxification, cell wall and cuticle biosynthesis, and osmoticum biosynthesis processes (Table 1) was quantified by qRT-PCR in leaf tissues of V/2xRL and V/4xRL challenged with water deficit. Quantitative data were normalized, and the resulting expression values were correlated with the microarray expression log (2) ratios. The linear regressions indicated a goodness of fit (R^2) of respectively 0.92 and 0.73 between both kinds of analyses (Supporting Information Fig. S2). The expression of a *Citrus* NCED1 candidate gene (Agustí *et al.* 2007), which is involved in ABA biosynthesis, was also investigated. Quantitative data obtained from down- and up-regulated genes were normalized and the resulting expression log (2) ratios are shown in Fig. 4. Stress/control ratios in the leaves of V/2xRL and V/4xRL trees exhibited similar trends (Fig. 4a) and allowed the microarray results to be confirmed, because the induction and repression patterns obtained with the microarray and qRT-PCR techniques were similar. Stress/control ratios were also obtained in roots with the same set of genes. As expected, genes involved in photosynthesis and cuticle biosynthesis were hardly expressed in V/2xRL and V/4xRL roots and are not presented (Fig. 4b). Genes that were up-regulated in response to stress in leaves were also up-regulated in roots. However, genes down-regulated in response to stress in leaves were mostly up-regulated in roots. Notably, 2xRL presented a more pronounced up-regulation of all tested genes in roots than 4xRL genotypes. The CsNCED1 candidate gene was not significantly up- or down-regulated in response to stress in the leaves of V/2xRL and V/4xRL trees, based on microarray data or RT-qPCR results (Fig. 4a). However, in stress conditions, CsNCED1 was up-regulated in V/2xRL roots (Fig. 4b). For the same set of genes, gene expression ratio of V/4xRL to V/2xRL leaves and roots was investigated in control and stress conditions. Under control conditions, the gene expression ratios of V/4xRL to V/2xRL for leaf samples ranged from –0.8 to 1.5, and most of them (14 over 20 genes) presented Log2 ratio close to 0 (Fig. 5a). Interestingly, the analysis of the expression ratios of V/4xRL to V/2xRL of the same genes in roots showed a dramatic up-regulation for all genes, except genes involved in photosynthesis and cuticle biosynthesis that were very little expressed and were not represented in Fig. 5a. A comparison of the V/4xRL and V/2xRL gene expression ratios in stressed leaves did not reveal large changes in expression, because the values were less than 1.5 for all of the genes (Fig. 5b). In stressed roots, the expression of genes was greater in V/4xRL than in V/2xRL, but the difference in expression was smaller than in control conditions (Fig. 5b).

DISCUSSION

What causes the lower g_s values measured in the leaves of V/4xRL trees in control conditions?

An investigation of the leaf morphology phenotype (leaf area and thickness) of Valencia orange trees grafted onto 2x and 4xRL rootstocks grown in control conditions did not show any significant difference between the two types of trees. Previous studies performed in polyploids showed that 4x citrus seedlings present lower rates of whole tree transpiration than 2x, which led to a reduced growth rate (Romero-Aranda *et al.* 1997; Allario *et al.* 2011). We also observed that in 4xRL seedlings, the reduction of g_s was associated with an increase in stomatal size and a decrease in stomatal density (Allario *et al.* 2011). Before pruning to generate homogenous trees, V/2xRL trees were taller than those of V/4xRL. Interestingly, the leaf g_s of V/4xRL was lower than that of V/2xRL

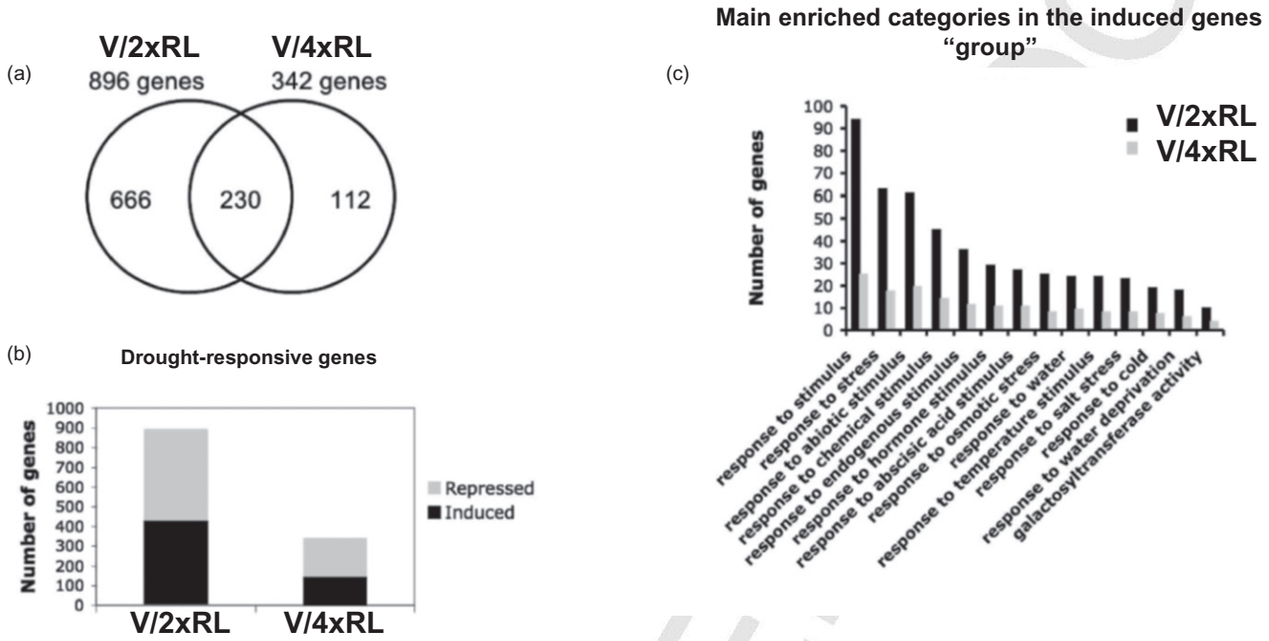


Figure 3. Microarray analysis. (a) The number of genes differentially expressed in the leaves of V/2xRL and V/4xRL trees. (b) Drought responsive genes repressed and induced in V/2xRL and V/4xRL trees subjected to water deficit. (c) Main enriched categories in groups of induced genes.

(Fig. 1a), but was not associated to any difference in stomatal size and density in control mature leaves (Table 2). It seems therefore that the reduced g_s values measured in the leaves of V/4xRL compared with V/2xRL cannot be explained by a change of the anatomy or the morphology of the 2x Valencia leaf whatever the ploidy of the rootstock is. In control conditions, the leaf RWC of both tree associations was not significantly different (Table 2), and most of the gene expression ratios of V/4xRL to V/2xRL for leaf samples were close to 1. Then, we postulate that V/4xRL and V/2xRL control trees were not experiencing any stress that may have led to a change of anatomy or morphology of the 2x Valencia leaves. Then, we hypothesize that the lower g_s values and lower growth rate observed in V/4xRL trees are caused by the 4x root system via a root-to-shoot signalling.

Investigations have shown that shoots may sense soil moisture status via the root-to-shoot translocation of a chemical signal before any detectable changes in shoot–water relations are produced (Blackman & Davies 1985; Gutschick & Simonneau 2002). Recently, investigations of the herbaceous perennial *Chamerion angustifolium* L. Holub suggested that the improved tolerance to water stress of 4x plants is associated with an increased efficiency of absorbing soil moisture before leaf water potentials that cause stomatal closure are reached (Maherali, Walden & Husband 2009). Regarding the plant water status, ABA is the main hormonal signal in root–shoot communication (Tardieu & Davies 1992; Dodd 2003, 2005). However, it has been shown that grafting a wild-type scion onto an ABA-deficient rootstock generally has no effect on stomatal conductance or whole plant transpiration rate, implying that wild-type shoots can synthesize enough ABA to control their water balance (Holbrook *et al*. 2002; Dodd *et al*. 2009). Our results showed that V/4xRL trees had a higher level of ABA than V/2xRL, not only in the leaves but also in the root (Fig. 2). As control trees were watered at field capacity to prevent any water deficit, we suggest that the higher ABA levels measured in leaves of V/4xRL originated in the root system and that ABA was transported to the shoot through the transpiration stream. Consequently, we hypothesize that this higher leaf ABA content may contribute to the regulation of stomatal conductance in the 4x genotype, minimizing the tree's water loss and decreasing its growth rate (Figs 1b and 6a). Recent experiments (Martin-Vertedor & Dodd 2011) also show that ABA supplied to the xylem of detached barley (*Hordeum vulgare*) shoots decreases shoot growth by 40% in a short-term (6 h) experiment. We can of course not exclude that the use of 4x rootstock may lead to other physiological changes such as the root hydraulic resistance or hormonal balance that may affect the growth rate of the tree.

Gene expression profiling demonstrated that V/2xRL plants were more affected by stress than V/4xRL

The purpose of monitoring gene expression in the clonal 2x Valencia orange variety was to characterize the impact of using a 4x rootstock compared with a 2x rootstock on gene expression in leaves without any interference of leaf polyploidy status. Besides the physiological evidence that V/4xRL trees are better adapted to drought than are V/2xRL trees (Fig. 1), the higher number of differentially expressed genes

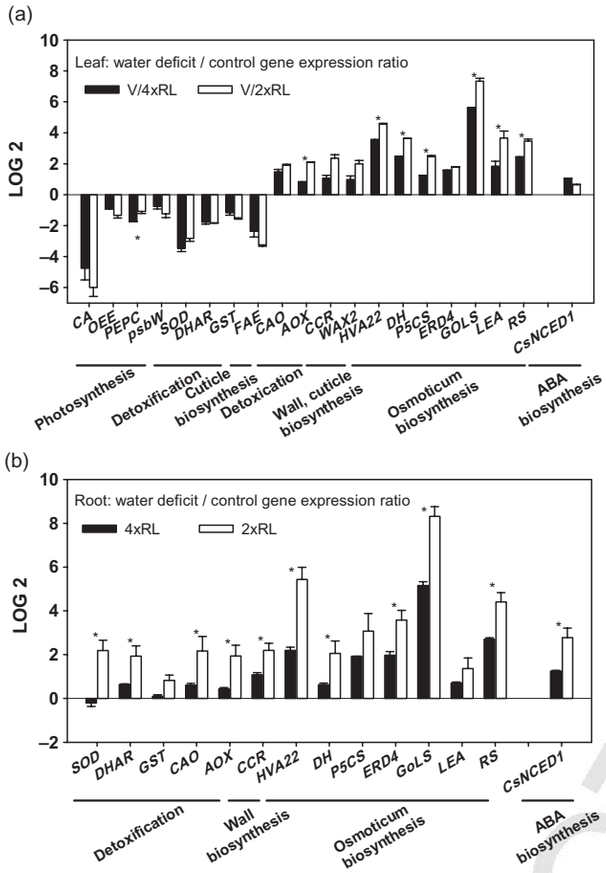


Figure 4. Gene expression, as determined by qRT-PCR, in the leaves and roots of V/2xRL and V/4xRL trees. (a) Gene expression in the leaves of V/2xRL and V/4xRL genotypes. Results are presented as a water deficit condition/control condition gene expression ratio. (b) Gene expression in the roots of V/2xRL and V/4xRL genotypes. Results are presented as a water deficit condition/control condition gene expression ratio. Vertical bars indicate the mean_ ± SE ($n = 3$). For each gene, * indicates a significant difference (t-test) between V/2xRL and V/4xRL genotypes ($P < 0.05$). Gene name and function are presented in Table 1.

involved in the response to water deficit (threefold greater in V/2xRL; Fig. 3) indicated that V/2xRL trees were responding more strongly to stress than V/4xRL. Indeed, as V/2xRL and V/4xRL trees did not present the same water consumption, trees were subjected to different soil water status when samples were harvested. Logically, V/4xRL trees, which have taken up less water, appeared less affected by stress than V/2xRL trees. As a consequence, the leaf water potential of V/2xRL was probably lower than V/4xRL. It can be hypothesized that the better tolerance observed in V/4xRL is associated to avoidance mechanisms (Verslues *et al.* 2006), because in control condition root anatomy and morphology of 2x and 4xRL seedlings were very different (Allario *et al.* 2011). Indeed, we observed that the growth of 2xRL was more vigorous than 4x, although roots of 4x plants were thicker and contained larger cells than 2x, which may have a large impact on cell-to-cell water exchanges leading to possible changes of hydraulic resistance in control and water deficit condition.

About 70% of the genes differentially expressed in the leaves of V/4xRL trees were also differentially expressed in V/2xRL, indicating that V/4xRL also responded to the drought treatment. This is evidenced by (1) the similar pattern of expression exhibited by the common set of genes differentially expressed between drought and control conditions in both genotypes (Supporting Information Fig. S1); and (2) the nature of the most important sets of genes induced in both V/2xRL and V/4xRL trees, which include functional categories such as 'response to ABA stimulus', 'response to osmotic stress' and 'response to water' (Fig. 3c and Supporting Information Table S3). Interestingly, clear differential responses were observed in the leaves of V/2xRL and V/4xRL trees, according to the sets of repressed genes. Functional categories mainly related to metabolic processes such as photosynthesis, carbon utilization and fatty acid biosynthesis were much more severely repressed in V/2xRL

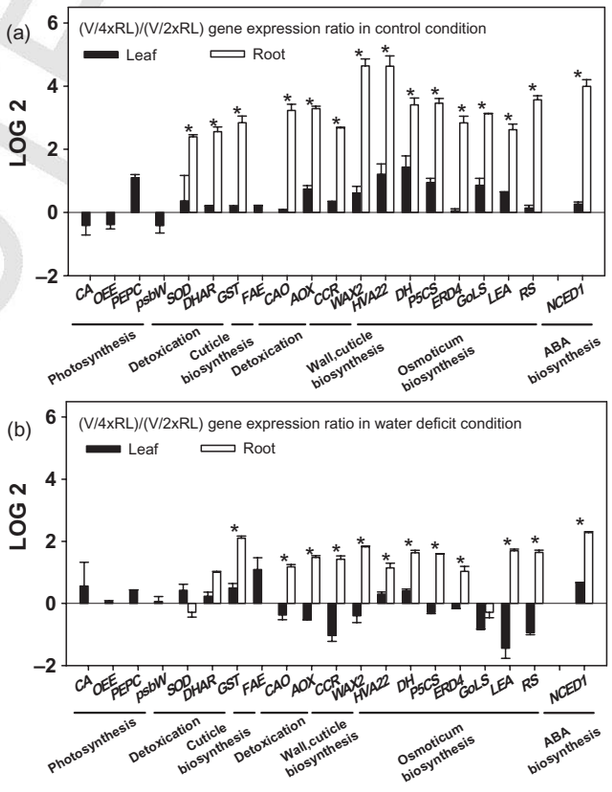
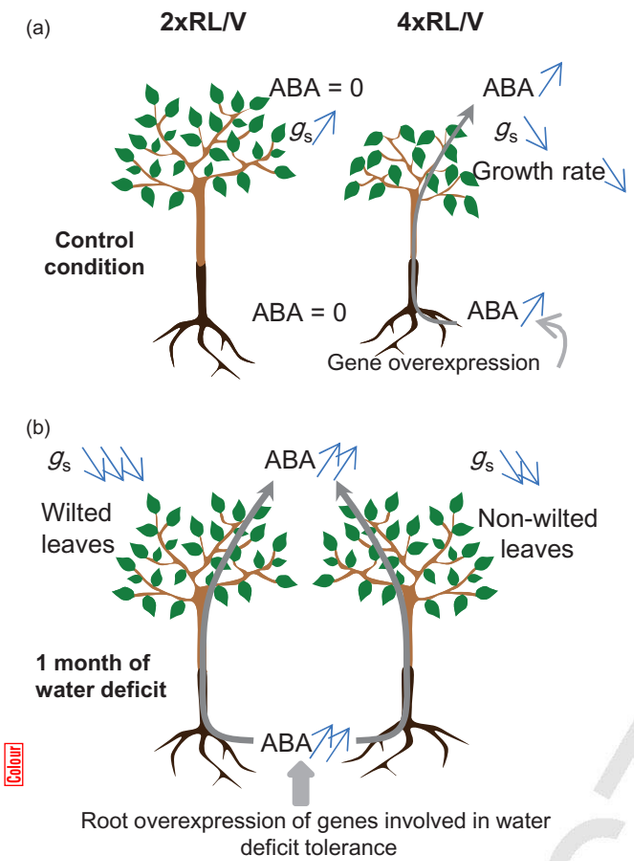


Figure 5. Gene expression, as determined by qRT-PCR, in the leaves and roots of V/2xRL and V/4xRL trees. (a) Gene expression in the leaves and roots of V/2xRL and V/4xRL genotypes grown in control conditions. Results are presented as the ratio of 4xRL/2xRL gene expression. (b) Gene expression in the leaves and roots of V/2xRL and V/4xRL genotypes in water deficit conditions. Results are presented as a 4xRL/2xRL gene expression ratio. Vertical bars indicate the mean_ ± SE ($n = 3$). For each gene, * indicates a significant difference (t-test) between the leaf and the root ($P < 0.05$). Gene name and function are presented in Table 1.



Colour

Figure 6. Schematic model of growth, g_s responses and root gene expression in V/2xRL and V/4xRL trees under non-stressed and water-stressed conditions. (a) In well-watered (control) conditions using rootstock/scion associations of the same age, V/2xRL presented greater vigour than V/4xRL. V/4xRL trees had higher root abscisic acid (ABA) contents than V/2xRL trees; ABA is transferred to the shoot, leading to lower g_s values and slower tree growth rate. Whatever the size of the tree is, the higher ABA contents in 4x roots correlated with the constitutive overexpression of numerous genes, such as *NCED1*, that are involved in ABA biosynthesis. (b) When V/2xRL and V/4xRL trees of the same size were subjected to water deficit, V/2xRL trees had lower g_s values than V/4xRL at the end of the experiment. In addition, wilted leaves were observed in V/2xRL because higher g_s and higher water consumption occurred in V/2xRL during the first days of the stress, demonstrating the increased tolerance to drought of 4x rootstock/scion associations.

than in V/4xRL trees. This may be linked to the repression of central metabolic processes, which can be considered to be secondary responses common to different stress factors. Inhibition of photosynthetic and carbon metabolism at the transcriptional level is known to be a general response of herbaceous (Xiong *et al*. 2001; Xiong, Li & Zhang 2006) and woody plants like *Citrus* (Forment *et al*. 2005; Christmann *et al*. 2007) to water deficit and osmotic stress. This differential response is likely due to a greater soil water deficit that suffered V/2xRL compared with V/4xRL trees and supports the hypothesis that water deficit is more efficiently avoided when citrus is grafted onto 4x rootstocks.

In addition, the inhibition of photosynthetic and carbon metabolism at the transcriptional level in water-stressed V/2xRL trees could be the consequence of the stronger reduction of stomatal conductance leading quickly to a decrease of photosynthesis and carbon metabolism (Figs 1a, 1b and 5).

What is the molecular origin of the improved tolerance of V/4xRL to water deficit?

4xRL arise from chromosome set doubling of nucellar cells (maternal tissue) (Cameron & Soost 1969). Consequently, in RL it is possible to compare the phenotypes and transcriptomes of 4x versus 2x genotypes immediately after the tetraploidization event. Spontaneous doubled diploid clones obtained from specific diploid citrus species are considered to be genetically identical to each other, with the same genome expression profile. Indeed, in autopolyploids genotypes including RL, gene expression changes in leaves were shown to be very limited when compared with the respective diploid parental (Stupar *et al*. 2007; Riddle *et al*. 2010; Allario *et al*. 2011; Li *et al*. 2012).

The study of differentially expressed genes obtained from microarray experiments contributed to the understanding of the observed responses of V/2xRL versus V/4xRL both in a quantitative and a qualitative manner. Quantitatively, the genetic response was stronger in V/2xRL in terms of total number of differentially expressed genes, suggesting a greater sensitivity to water deficit in plants grafted onto the diploid rootstock. Qualitatively, the response of V/4xRL affected more specifically the expression of genes that are directly involved in abiotic stress acclimatization processes. This statement is based on the fact that the relative abundance of functional categories specifically induced by abiotic (ABA, drought or osmotic) stimuli was more important in V/4xRL than in V/2xRL trees (Supporting Information Table S5). We recently reported a similar genomic approach, comparing global gene expression in the leaves of 2xRL versus 4xRL seedlings under control conditions (Allario *et al*. 2011), showing that large changes in anatomy and physiology between V/2xRL and V/4xRL were not associated with large changes in leaf gene expression. We observed that 'response to ABA stimulus' was a relevant functional category overexpressed in the leaves of 4xRL trees compared with those of 2xRL under control conditions. On the other side, repression of primary metabolic processes like photosynthesis and carbohydrate metabolism can be considered a less specific or secondary response common to other plant species and perturbing factors (Kawasaki *et al*. 2001; Ozturk *et al*. 2002; Seki *et al*. 2002; Sahi *et al*. 2003; Khelil, Menu & Ricard 2007; 14 Brumós *et al*. 2009), which is indicative of a plant that is experiencing a stressful situation. According to microarray results, this phenomenon is clearly produced in diploid-grafted rather than autotetraploid-grafted plants. Altogether, these results support the notion of a preaclimatacion of autotetraploid-grafted plants that make the trees more tolerant water deficit.

Franck (1980) investigated the water deficit tolerance of wheatgrass polyploids. This study reported the photosynthetic activity in control and water deficit conditions of hexaploid (6x) and 4x genotypes that exhibited increased drought tolerance levels that were associated with a higher capacity to fix CO_2, yet at least in the early stages of drought stomatal conductance closed more sensitively in response to soil drying (Xiong *et al*. 2006) showed that 6x wheat lines were more tolerant to water deficit than 4x, and that 4x genotypes were more tolerant than 2x. When subjected to a progressive water deficit, 6x wheat lines decreased their stomatal conductance before the 4x and 2x wheat lines did. In these studies, polyploids were able to maintain a constant leaf RWC longer than 2x plants, indicating a more sensitive response to root-to-shoot chemical signalling (likely ABA) rather than to a hydraulic signal (Xiong *et al*. 2001). Citrus *CsNCED1*, which is the ortholog of *AtNCED3*, was shown to be up-regulated in response to stress (Agustí *et al*. 2007) and was dramatically up-regulated in 4x roots relative to 2x roots in control conditions (Fig. 5a), which is in agreement with the greater ABA contents we measured (Fig. 2). The increased expression of stress-responsive genes in the roots of 4x trees compared with those of 2x trees (Figs 5a and 6a) suggests that V/4xRL citrus trees are better able to withstand water shortage than are V/2xRL, as previously proposed (Allario *et al*. 2011). This is in contrast to the limited change in gene expression observed in the leaves of 2x and 4xRL seedlings under control conditions (Allario *et al*. 2011), suggesting that differential gene expression occurs between the leaves and roots of V/2xRL and V/4xRL trees. Recently, Li *et al*. (2012) tested 2x and 4x *A. thaliana* with respect to the gene expression under control compared with mannitol or glucose conditions. Interestingly, the overall changes of gene expression between 2x and 4x in mannitol treatment were milder than in glucose treatment. They conclude that the response of the 4x plants to glucose treatment is endogenously different from the 2x plants. Indeed, these authors observed that during root development, the difference of root cell size and cell number between 2x and the 4x under the glucose treatment was significant, but that under mannitol was not. The two water deficit experiments we performed in citrus lasted for 11 and 24 d, respectively. The same phenotype of better adaptation of V/4xRL compared with V/2xRL was observed in both experiments. Because citrus are slow-growing plants, we can hypothesize that no large changes of cell size and anatomy induced by stress may have occurred in roots during the experiments. Therefore, the higher ABA content measured in roots of V/4xRL in control condition would be a direct consequence of the constitutive overexpression of *CsNCED1* in 4x roots.

Under water deficit conditions, the gene expression ratio of stressed leaves to control leaves was not significantly changed, regardless of the ploidy status of the rootstock, except for a few genes that were expressed at greater levels in V/2xRL. As expected, the genes of water-stressed trees showed a greater level of overexpression in the roots of V/2xRL trees than of V/4xRL, confirming that a variety grafted onto a 2x rootstock is more sensitive to drought than when grafted onto a 4x rootstock (Fig. 6b).

Our results support the interpretation that long-distance ABA signalling from root to shoot leads to important changes in shoot physiology when plants are experiencing water deficit, although ABA signalling may not be sufficient by itself (Christmann *et al*. 2007) and may be modified by other factors (Dodd *et al*. 2009). We could not find other studies in which this type of signalling has been described in two genotypes (2x and 4xRL roots) that are genetically identical but with a different level of ploidy. In 4xRL, this root-to-shoot signalling is mediated by a constitutive differential expression of several genes, including the *CsNCED1* gene, which led to increased root-to-shoot transfer of ABA, and consequently was at the origin of greater regulation of gas exchange of scions grown on 4x root genotypes. Therefore, the use of autotetraploid citrus genotypes as rootstock is a powerful technique for demonstrating the importance of root-to-shoot signalling and the consequences on plant physiology. Further investigations are now needed to better understand the differential regulation of genes in autotetraploid roots compared with diploid roots.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Gene expression patterns in leaves of V/2xRL and V/4xRL.

Figure S2. Correlation between microarray and qRT-PCR expression analyses.

Table S1. Genes differentially expressed in the diploid (V/2xRL) genotype in response to water deficit.

Table S2. Genes differentially expressed in the autotetraploid (V/4xRL) genotype in response to water deficit.

Table S3. Significantly enriched functional categories in the group of genes induced by water deficit.

Table S4. Significantly enriched functional categories in the group of genes repressed by water deficit in V/2xRL and V/4xRL trees.

Table S5. Relative abundance of induced genes in different functional categories.

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