

Comparative study of putative 9-*cis*-epoxycarotenoid dioxygenase and abscisic acid accumulation in the responses of Sunki mandarin and Rangpur lime to water deficit

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Abstract Abscisic acid is a plant hormone that participates in essential plant physiological processes, especially during adaptation to many environmental stresses, such as water deficit. The relationship between ABA accumulation and the expression of putative carotenoid cleavage dioxygenase (CCD) genes was investigated in the pot-cultivated leaves and roots of the ‘Rangpur’ lime and ‘Sunki Maravilha’ mandarin plants. Transpiration, stomatal resistance and leaf growth were evaluated when these genotypes were subjected to continuous water deficit. Under water deficit conditions, the ‘Rangpur’ lime extracts used greater amounts of water when compared to the ‘Sunki Maravilha’ plants, which reached the greatest stomatal resistance 5 days before ‘Rangpur’ lime. When subjected to water deficit, the roots and leaves of ‘Sunki Maravilha’ showed a progressive increase in ABA accumulation; however, in ‘Rangpur’ lime, alternations between high and low ABA concentrations were observed. These results suggest a retroactive feeding regulation by ABA. In ‘Rangpur’ lime the

NCED2, NCED3 and CCD4a genes were expressed at the highest levels in the roots, and NCED5 was highly expressed in the leaves; in ‘Sunki Maravilha’, the NCED2 and NCED5 genes were most highly expressed in the roots, and NCED2 was most highly expressed in the leaves. However, for both genotypes, the transcription of these genes only correlated with ABA accumulation during the most severe water deficit conditions. The ‘Rangpur’ lime behaved as a vigorous rootstock; the leaf growth remained unaltered even when water was scarce. However, ‘Sunki Maravilha’ adaptation was based on the equilibrium of the response between the root and the aerial tissues due to water restriction. The use of the Sunki mandarin in combination with a scion with similar characteristics as its own, which responds to water deficit stress by accumulating ABA in the leaves, may display good drought tolerance under field conditions.

Keywords ABA · NCEDs · Drought stress · Drought tolerance · *Citrus* spp.

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Abbreviations

ABA Abscisic acid
FTSW Fraction of transpirable soil water
NCED 9-*cis*-Epoxy-carotenoid dioxygenase
NTR Normalised transpiration rate
TDR Time domain reflectometry

Introduction

The prospective outcomes of global climate change are changing faster than anticipated, and they predict that the world’s dry regions will increase in the near future [1]. One predicted problem is the overuse of agricultural water

resources, which will decrease plant growth, survival and crop yield potential [2, 3]. Droughts tend to promote changes in plant growth and development; these conditions halt aboveground growth, promoting leaf abscission and increasing the mass of the root system to promote the acquisition of water in deeper soils [4]. These processes have been associated with increased abscisic acid (ABA) contents, a key phytohormone that promotes growth and development in plants [5]. Studies regarding hormonal regulation in citrus plants have proposed that ABA works as a mediator between environmental changes and plant responses to different types of stress [6]. Therefore, it is important to consider that ABA is a phytohormone that is involved in different developmental processes, including seed formation, dormancy and germination and plant defence responses to biotic and abiotic stresses, such as drought, cold and salinity [7].

The primary steps of ABA biosynthesis occur in the chloroplasts and plastids and are catalysed by the 9-*cis*-epoxycarotenoid dioxygenase (NCED) enzyme [8] to produce xanthoxin, the direct precursor to ABA. One of the major advancements in the understanding of ABA biosynthesis regulation was the identification of NCED, which was first described during the characterisation of the maize (*Zea mays*) mutant *viviparo14* (*vp14*) [9]. *notabilis*, an ABA deficient mutant tomato, was later discovered to also contain a mutation in the *Vp14* gene (LeNCED1) [10]. The *NCED* gene was later cloned in several species, such as bean (*Phaseolus vulgaris*) [11], avocado (*Persea americana*) [12] and *Arabidopsis thaliana* [9, 13, 14].

NCED comprises a family of homologous genes in all of the analysed species. After 9-*cis*-epoxycarotenoid is cleaved in plastids, the resulting compound xanthoxin is converted into ABA in the cytosol. Studies with *P. vulgaris* have confirmed that NCED is expressed during water deficiency, clearly demonstrating the relationship between the expression of mRNA (PvNCED1), protein (NCED) and ABA levels in dehydrated leaves and roots. These results implicate the regulatory role of NCED in ABA biosynthesis during water deficiency [11]. The current study evaluated the physiological mechanisms of drought tolerance, leaf area ratio, water potential, stomatal resistance, ABA levels and profile of the NCED multigenic family (NCED1, NCED2, NCED3, NCED5 and CCD4a) with respect to their involvement in ABA biosynthesis in ‘Rangpur’ lime and ‘Sunki Maravilha’ mandarin. The ‘Rangpur’ lime, as well as other varieties of this lime, is drought tolerant, whereas the ‘Sunki Maravilha’ mandarin shows greater susceptibility to water stress in comparison to the former, confirmed by the accentuated curling of the leaf presented by this tangerine during water stress.

Materials and methods

Plant material and submission to water deficiency stress

Citrus limonia Osb (Rangpur Lime) and *Citrus sunki* (‘Sunki Maravilha’ mandarin) rootstocks were used in the study. The rootstocks were selected according to their drought tolerance; Rangpur Lime is tolerant, and ‘Sunki Maravilha’ mandarin is susceptible [15]. The selected plants were 6 months old, had an average of 15–20 leaves and were maintained in seedling nursery. The plants were first transplanted into 15 L pots that contained growth media composed of Plantmax (Eucatex Agro.), washed sand and clay in a ratio of 2:1:1. The fertiliser was applied to the aerial portion of the plant. The plants were transferred to the greenhouse and maintained under controlled humidity and air temperatures (70–80 % relative humidity and 25–30 °C).

Forty-five days after transplantation, the seedlings were divided into 2 groups as follows: (i) the control treatment, in which the plants were permanently irrigated near field capacity and (ii) treatment under water deficiency, in which the plants were subjected to the absence of irrigation. The pots were capped at the top with aluminium foil to minimise evaporative water loss. The water deficiency application period was 40 days; after this point, the physiological analysis was performed to determine the collection time for each genotype.

Water deficiency occurred gradually as the remaining soil water was depleted. The experiment was performed in a completely randomised design, including 4 replicates of 1 control treatment and 3 replicates each of 3 treatments under water deficiency. The humidity was monitored by measuring the soil water ($\text{m}^3 \text{m}^{-3}$) content using a TDR (time domain reflectometry) probe. To calculate the plant transpiration ($\text{L m}^{-2} \text{leaves day}^{-1}$), the soil moisture differences were calculated between the early morning (θ_i) and late afternoon (θ_f) values; the volume of the soil explored was known (V_s), and the total leaf area ($LA - \text{m}^2$) was defined as follows: $(\theta_i - \theta_f)V_s/LA$.

The relationship between transpiration and soil moisture was estimated for each genotype by relating the normalised transpiration rate (NTR) to the fraction of transpirable soil water (FTSW) according to the method of Sinclair and Ludlow [16]. The NTR was calculated by dividing the daily transpiration rate of each plant in the water deficiency group by the average transpiration rate of the control plants (Cont) for each genotype. The FTSW was calculated by determining the difference between the water humidity at the beginning of the day (w_d) and the water humidity at the end of the treatment (w_f); this difference was divided by

the difference between the water humidity at the beginning of the treatment (w_i) and the water humidity at the end of the treatment (w_f).

$$FTSW = \frac{wd - wf}{wi - wf}$$

Based on the soil moisture data ($\text{m}^3 \text{m}^{-3}$), three time periods were selected for the collection of leaf and root samples from the plants under water deficiency; one time period was selected for the control plants. The collection times were based on the soil water humidity levels as follows: 0.20–0.19, 0.17–0.16 and 0.15–0.14 $\text{m}^3 \text{m}^{-3}$. For the control plants, the soil water humidity value was 0.29–0.28 ($\text{m}^3 \text{m}^{-3}$). The plant materials were cut with a scalpel blade, wrapped in aluminium foil and immediately frozen in liquid nitrogen; the materials were then stored at -80°C for later analyses.

Determination of leaf area

Young leaves were selected from each individual plant. The length and width were measured daily to establish their growth rate. When new leaves emerged, the previously analysed leaves were replaced, and the evaluations were continued from that date forward. When the plant ceased to release new leaves, the growth rate was considered to be 0 (zero). The leaf area was determined by multiplying the length by the width. This result was multiplied by an adjustment factor of 0.72.

Stomatal resistance

Leaf stomatal resistance was measured with a portable porometer AP4 (Delta-T Devices, Newmarket, UK). The measurements were taken daily under ambient light and CO_2 conditions between the hours of 09 h 30 min and 1 h 30 min during the entire water deficiency period; the water deficiency period for Rangpur Lime was 4 days less than that of the mandarin ‘Sunkei Maravilha’.

Leaf water potential

Leaf water potential was determined between 4 and 5 h with a Scholander-type pressure pump (m670, Pms Instrument Co., Albany, USA). The leaves were excised with a scalpel blade, and data were immediately collected.

ABA analysis

ABA samples were extracted from 500 mg samples that were composed of lyophilised plant material; leaf and root samples were analysed from both genotypes. ABA was extracted as previously described [17] with some

modifications. The samples were macerated and extracted in acetone overnight at 4°C . The extract was sonicated at low amplitude for 1 min. The samples were then filtered and evaporated. The residue was dissolved in 3 ml of ethyl acetate and *n*-hexane (1:9, v/v); this step was repeated three times to obtaining of a pool of the aqueous phase. Next, 3 ml of water was added to the samples, which were then evaporated in the rotary evaporator. The samples were filtered through a nylon membrane (0.22 μm). The pH was then adjusted to 2.5 with 1 M HCl, and the samples were saturated in 1/3 ethyl acetate (v/v). The organic phase was collected and evaporated, and the residue was dissolved in methanol.

An Agilent chromatograph equipment (Palo Alto, CA, USA) 1200 series was used; it contained a G1311A quaternary pump, a G1367B self-injection apparatus, G1322A a degasificator, and a G1315D diode array detector. The analyses were performed using an Agilent XDB-C18 eclipse column (250 \times 4.6 mm; particle size 5 μm), eluted with water (solvent A) and methanol (solvent B) under a flow of 1 ml min^{-1} . The elution involved a gradient starting with 10 % solvent B with a linear change towards 100 % solvent B over 10 min. The detection was carried out from 190 to 400 nm.

The quantification was performed using the HPLC-UMS, which consists of the previously described HPLC coupled to an API 2000 mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionisation source (ESI) and a triple-quadrupole type analyser (QqQ). The equipment was operated using the ESI source in negative polarity. The analytical parameters were optimised. The spray voltage was at $-4,500$, drying gas 10, and nebuliser gas 50. Nitrogen was used as the collision gas. The data acquisition and processing were performed using the Analyst 1.5.1 software program (Applied Biosystems).

RNA extraction and cDNA synthesis

After the leaf water potential measurements were performed, the plant material to be examined (leaves and roots and total RNA samples) were isolated using the RNAqueous Kit (Ambion, Inc.) according to the manufacturer’s instructions. The RNA quality and integrity were assessed by analysis in a 1.5 % agarose gel. The samples were treated with DNase following extraction with the Turbo DNA-free Kit (Ambion, Inc.) according to the manufacturer’s instructions (1 μL of DNase). The absence of genomic DNA contamination was confirmed by performing negative PCR using primers for the β -actin gene. The reverse transcription reaction was performed using the RETROscript kit (Ambion Inc.) according to the manufacturer’s instructions.

Gene expression analysis

The abundance of mRNA transcripts was analysed by real-time PCR using an ABI Prism 7500 and the “Sequence Detection System” program (Applied Biosystems). The β -actin (ACT) gene was amplified with the target gene as an endogenous control to normalise the expression between the different samples.

The real-time PCR reactions were performed in triplicate in 20 μ L reaction volumes; each reaction contained 75–100 ng of cDNA, 1 μ L of primer (F + R) in the concentration of 500 nM, 10 μ L of Maxima[®] SYBR Green/ROX qPCR Master Mix (2 \times) (Fermentas, EUA) and \sim 8 μ L of sterile Milli-Q water. The amplification was performed according to the following steps: (1) activation of *Taq* DNA polymerase at 50 $^{\circ}$ C for 2 min, (2) initial denaturation at 95 $^{\circ}$ C for 10 min, (3) denaturation at 94 $^{\circ}$ C for 15 s, (4) annealing at 60 $^{\circ}$ C for 30 s and (5) extension at 60 $^{\circ}$ C for 1 min. Steps 3–5 were repeated for 45 cycles.

The comparative method of C_t ($\Delta\Delta C_t$) was used to quantify the gene expression, and the data were derived from three biological repetitions. Non-cDNA (NTC) control reactions were also used in all of the experiments. The Dissociation Curve software program v. 1.0 (Applied Biosystems, EUA) was used to verify that only one PCR product was generated by the amplification. A Student's *t* test was used to compare the expression values between the control and stress treatments.

The primers used in the experiments are described in Table 1 [6, 18, 19].

Results

Induction of water deficiency

Changes in transpiration in the evaluated genotypes were observed for the plants subjected to water restriction and

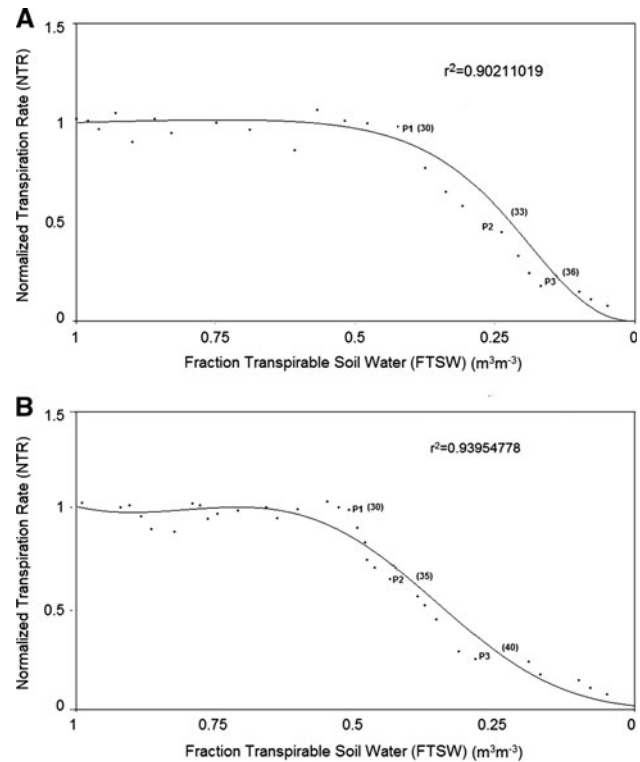


Fig. 1 Response curve of the normalised transpiration rate (NTR) according to the fraction of transpirable soil water (FTSW) in ‘Rangpur’ lime plants (a) and the mandarin plant ‘Sunki Maravilha’ (b). P1, P2 and P3 indicate the periods at which the samples were collected followed by the number of days that had elapsed from beginning of the experiment

experiencing changes in FTSW. The ‘Rangpur’ lime plants subjected to water deficiency displayed FTSW and NTR decreases of \sim 20 % with respect to the available water (FTSW of 0.19 and NTR of 0.24) (Fig. 1). When the mandarin plant ‘Sunki Maravilha’ was grown under water deficiency conditions, the FTSW and NTR values were

Table 1 Sequence of primers used in qRT-PCR experiments

Primer	Sequence	Reference
β -actina	F: 5'-TTAACCCCAAGGCCAACAGA-3' R: 5'-TCCCTCATAGATTGGTACAGTATGGAC-3'	[18]
NCED 1	F: 5'-GACCAGC AAGTGGTGTTCAA-3' R: 5'-AGAGGTGGAACAGGAGCAA-3'	[19]
NCED 2	F: 5'-GGTGCCAACCCATTATTCGA-3' R: 5'-GCCGTCACCGTCAAAGAAAT-3'	[19]
NCED 3	F: 5'-GGAGAATGAGGATGATGGCTAC-3' R: 5'-CTTTCGCGCTTATGAACGTG-3'	[6]
NCED 5	F: 5'-CTTCCAACGAAGT CCATAG-3' R: 5'-GGATTCCATTGTGATTGCTG-3'	[6]
CCD 4a	F: 5'-GCCATTATTTGTGCCAAGG-3' R: 5'-GATTTGGTGATTGGCATCC-3'	[6]

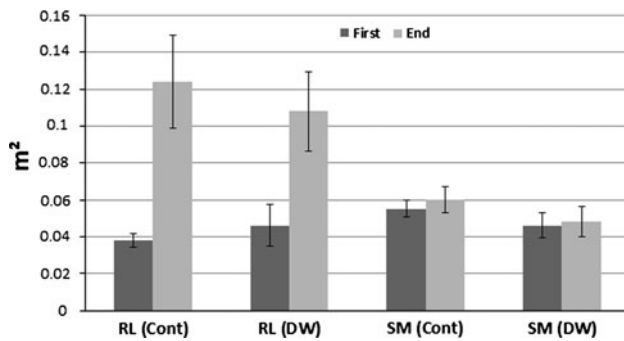


Fig. 2 Leaves of the control plants (Cont) and plants subjected to water deficiency (DW) from ‘Rangpur’ lime (RL) and the mandarin plant ‘Sunki Maravilha’ (SM) during the experimental period. (*) indicates that no significant differences were observed between the treatments

significantly reduced to $\sim 30\%$ of the available water (FTSW of 0.33 and NTR of 0.27).

Leaf area

When ‘Rangpur’ lime plants were examined 40 days after being subjected to water deficit, the leaf area per plant was $\sim 0.11\text{ m}^2$; the plants that were not subjected to a water deficit had a leaf area per plant of $\sim 0.12\text{ m}^2$ (Fig. 2). A different profile was observed in the ‘Sunki Maravilha’ mandarin because at the end of the experiment, the average control plant leaf area was 0.06 m^2 , and that of the plants subjected to water deficit was 0.041 m^2 . The ‘Rangpur’ lime and ‘Sunki Maravilha’ mandarin plants did not display statistically relevant differences when compared to the control plants and the plants subjected to water deficit at the end of the experiment. However, a difference was observed in the growth rates between the genotypes; the ‘Rangpur’ lime growth rate was high, and the ‘Sunki Maravilha’ mandarin growth rate was low.

Stomatal resistance

Many plants increase their stomatal resistance to conserve water because higher stomatal resistance confers reduced transpiration and, consequently, reduced water loss [20]. Stomatal resistance increased gradually during the entire deficiency period and was related to the FTSW data (Fig. 3) because as FTSW is reduced, stomatal resistance apparently increases. The ‘Sunki’ mandarin reached its maximum point of stomatal resistance when the soil fraction of transpirable water was $0.33\theta\text{ m}^3\text{ m}^{-3}$, which was higher than the value for ‘Rangpur’ lime ($0.19\text{ m}^3\text{ m}^{-3}$).

When comparing the treatments with the daily stomatal resistance values of the control plants and the plants subjected to water deficiency (Fig. 4), the ‘Rangpur’ lime FTSW was $0.20\text{--}0.19\text{ m}^3\text{ m}^{-3}$, and its mean stomatal resistance was

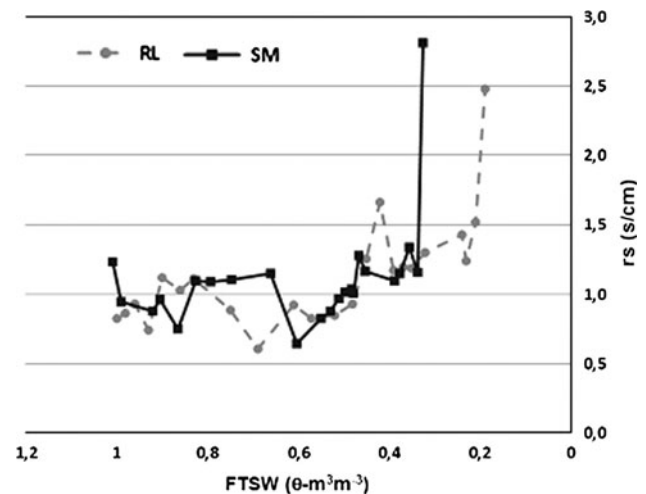


Fig. 3 Stomatal resistance in relation to the fraction of transpirable soil water in ‘Rangpur’ lime plant and the mandarin plant ‘Sunki Maravilha’ subjected to water deficiency (DH)

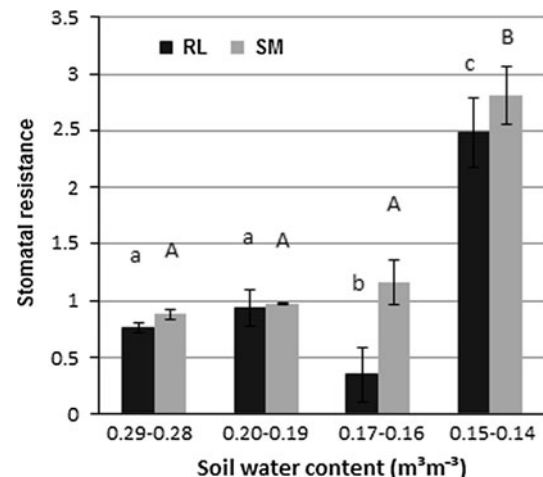


Fig. 4 Plant stomatal resistance (gs) according to the soil water content ($\text{m}^3\text{ m}^{-3}$) characterised by TDR for ‘Rangpur’ lime and the mandarin plant ‘Sunki Maravilha’. Tukey’s test ($p \leq 0.05$)

$0.93\text{ mmol cm}^{-2}\text{ s}^{-1}$. When the soil water decreased to $0.17\text{--}0.16\text{ m}^3\text{ m}^{-3}$, the plants displayed a slightly increased stomatal resistance of $1.20\text{ mmol cm}^{-2}\text{ s}^{-1}$. At $0.15\text{--}0.14\text{ m}^3\text{ m}^{-3}$, this increase was more pronounced, reaching a stomatal resistance of $2.48\text{ mmol cm}^{-2}\text{ s}^{-1}$. This increased stomatal resistance is related to the higher water deficiency; in the same period, the mean stomatal resistance of the control plants was $0.98\text{ mmol cm}^{-2}\text{ s}^{-1}$.

When the mandarin plant ‘Sunki Maravilha’ was grown in a soil with a water content of $0.20\text{--}0.19\text{ m}^3\text{ m}^{-3}$, the mean stomatal resistance of the plants’ leaves was $0.97\text{ mmol cm}^{-2}\text{ s}^{-1}$; this value was similar to that of the plants in which the water content was $0.29\text{--}0.28\text{ m}^3\text{ m}^{-3}$. When the soil water content progressively decreased to $0.17\text{--}0.16$

and $0.15\text{--}0.14\text{ m}^3\text{ m}^{-3}$, the mean stomatal resistance increased to 1.15 and $2.81\text{ mmol cm}^{-2}\text{ s}^{-1}$, respectively, indicating a negative correlation between the soil water content and stomatal resistance.

Leaf water potential (ψ_{WF})

In the control plants of both cultivars, the leaf water potential was always higher than -0.25 MPa. Statistically, the water potentials of the plants grown under water deficiency (soil water contents of $0.20\text{--}0.19$ and $0.17\text{--}0.16\text{ m}^3\text{ m}^{-3}$) were not significantly different ($p < 0.05$) than those of the control plants ($0.29\text{--}0.28\text{ m}^3\text{ m}^{-3}$). However, when the soil water content was $0.15\text{--}0.14\text{ m}^3\text{ m}^{-3}$, the cultivars displayed water potential values that were significantly different from the controls ($p < 0.05$); ‘Rangpur’ lime was -1.43 MPa, and the mandarin plant ‘Sunki Maravilha’ was -1.3 MPa (Fig. 5).

ABA analysis

The ABA contents in the ‘Rangpur’ lime and ‘Sunki Maravilha’ plants are shown in Fig. 6. The ABA levels in the ‘Sunki Maravilha’ leaves were higher than those observed in the roots. The leaf and root ABA content in the mandarin plant ‘Sunki Maravilha’ gradually increased, and this increased correlated with the reduced soil water content. However, when the soil water content reached $0.15\text{--}0.14\text{ m}^3\text{ m}^{-3}$, the ABA content of increased $\sim 80\%$ was verified in relation to the soil water content of $0.17\text{--}0.16\text{ m}^3\text{ m}^{-3}$ in the leaves and was below the limit of quantification in the roots. However, a clear,

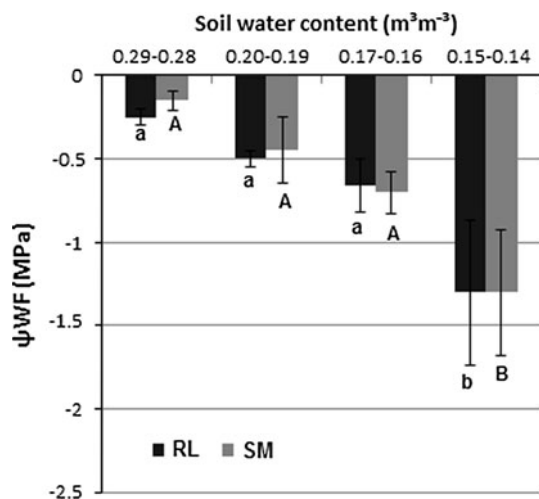


Fig. 5 Leaf water potential (ψ_{WF}) in ‘Rangpur’ lime plants and the mandarin plant ‘Sunki Maravilha’ according to the soil water content ($\text{m}^3\text{ m}^{-3}$) as characterised by TDR. The means followed by the same letter in SM, uppercase and lowercase in RL, displayed no significant differences according to Tukey’s test ($P \leq 0.05$)

gradual trend for ABA accumulation was not observed for ‘Rangpur’ lime when the soil water content decreased. It was confirmed that ABA can be measured in ‘Rangpur’ lime roots grown in all soil water content conditions; ABA accumulation peaks were observed at $0.20\text{--}0.19$ and $0.15\text{--}0.14\text{ m}^3\text{ m}^{-3}$. In the ‘Rangpur’ lime leaves, ABA accumulation peaks were observed when the soil water contents were $0.20\text{--}0.19$ and $0.15\text{--}0.14\text{ m}^3\text{ m}^{-3}$; ABA could not be quantified when the soil water contents were $0.29\text{--}0.28$ and $0.17\text{--}0.16\text{ m}^3\text{ m}^{-3}$.

Gene expression analysis

The cleavage of 9-*cis*-xanthophyll to xanthoxin represents a key step in plant ABA biosynthesis; this cleavage is mediated by the 9-*cis*-epoxycarotenoid dioxygenase (NCED) subfamily. To assess the importance of the carotenoid cleavage dioxygenase (CCD) genes in citrus plants, the relative abundance of the NCED1, NCED2, NCED3, NCED5 and CCD4a genes was determined. These genes may be involved in ABA biosynthesis during stress induced by water deficiency in the roots and leaves of tolerant and susceptible plants. The expression of each gene under normal conditions (without stress induction by water deficiency control) was determined as 1 and used to determine the relative expression of each gene under water deficiency stress conditions (Fig. 7). No specific expression pattern was observed between the leaves and roots within and among the studied genotypes. In the leaves and roots of both genotypes, the expression of the NCED1, NCED2, NCED3 and CCD4a genes was inhibited (down-regulated) when the soil water content decreased to $0.20\text{--}0.19$ and $0.17\text{--}0.16\text{ m}^3\text{ m}^{-3}$. The NCED5 gene behaved similarly to the other NCED family members; however, in the mandarin ‘Sunki Maravilha’ roots, no transcriptional decrease of this gene was observed when the soil water content was $0.20\text{--}0.19\text{ m}^3\text{ m}^{-3}$.

In the ‘Rangpur’ lime roots grown in soil with a water content of $0.15\text{--}0.14\text{ m}^3\text{ m}^{-3}$, NCED2 and NCED3 transcription increased approximately 12-fold, and that of CCD4a increased approximately 18-fold; however, the transcription of NCED1 and NCED5 was virtually zero. In the ‘Rangpur’ lime leaves that were grown with a soil water content of $0.15\text{--}0.14\text{ m}^3\text{ m}^{-3}$, the transcription of NCED5 increased approximately 12-fold, and that of the other NCED family members decreased. These results suggest that the NCED2, NCED3 and CCD4a genes play a role in ABA biosynthesis in ‘Rangpur’ lime roots subjected to water deficiency stress, while the NCED5 gene is related to ABA biosynthesis in ‘Rangpur’ lime leaves subjected to water deficiency stress. When the soil water content decreased to $0.15\text{--}0.14\text{ m}^3\text{ m}^{-3}$, the NCED2 and NCED5 gene expression levels increased approximately 50 and 18-fold, respectively, in the mandarin plant ‘Sunki Maravilha’ roots.

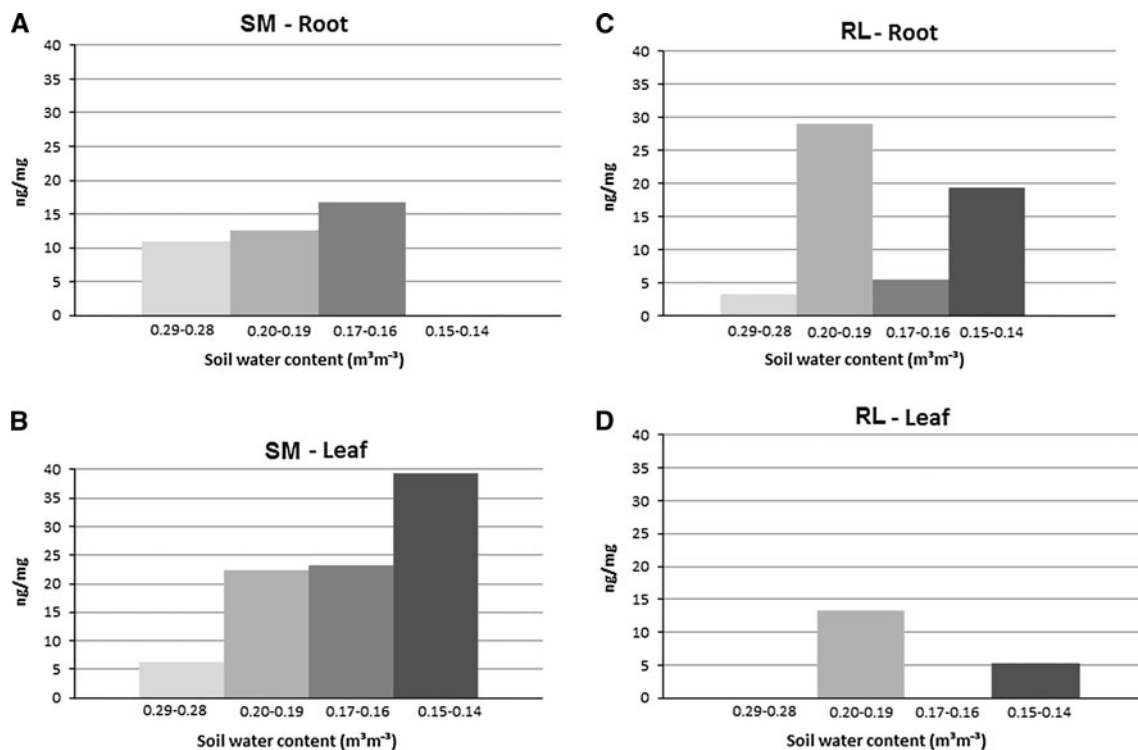
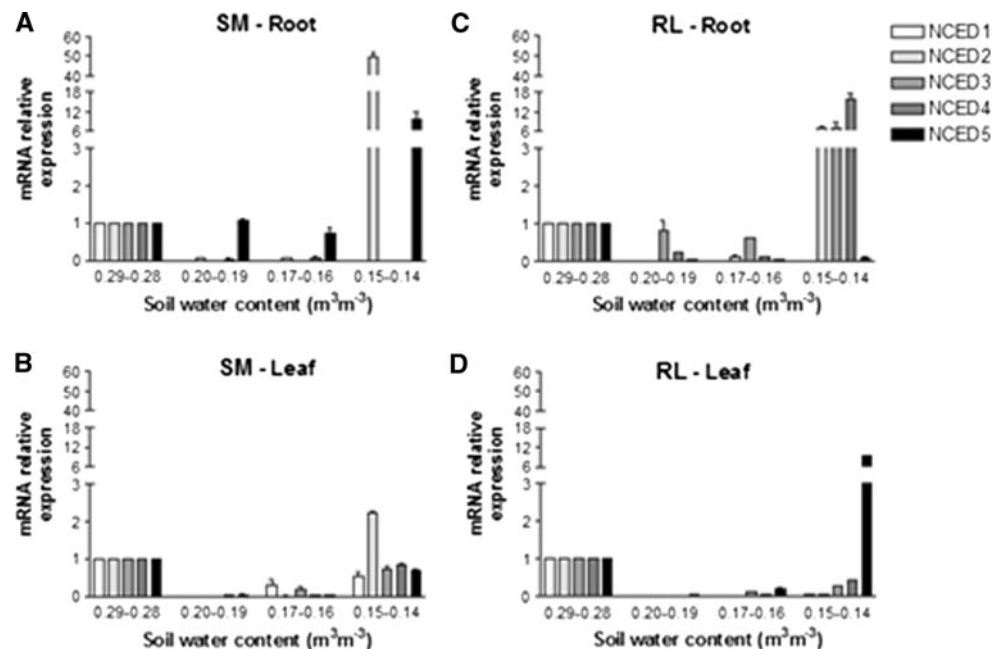


Fig. 6 ABA contents (ng mg^{-1} of dry weight) in different soil water levels ($\text{m}^3 \text{m}^{-3}$) in the genotypes ‘Rangpur’ lime and ‘Sunki Maravilha’. **a** ABA contents in the roots of the mandarin plant

‘Sunki Maravilha’, **b** ABA contents in the leaves of the mandarin plant ‘Sunki Maravilha’, **c** ABA contents in the roots of Rangpur Lime and **d** ABA contents in the leaves of Rangpur Lime

Fig. 7 Relative leaf and root expression levels of genes encoding the enzymes 9-*cis*-epoxycarotenoid dioxygenase/carotenoid cleavage dioxygenase (*NCED1*, *NCED2*, *NCED3*, *NCED5* and *CCD4a*) in ‘Rangpur’ lime plants and the mandarin plant ‘Sunki Maravilha’; the plants were grown under control conditions and under water deficiency from different soil water content levels ($\text{m}^3 \text{m}^{-3}$). β -actin gene expression was used as a normaliser. The data represent the mean \pm SE (standard error) of three experimental replicates



Similarly, when the soil water content decreased from from 0.17–0.16 to 0.15–0.14 $\text{m}^3 \text{m}^{-3}$ in the mandarin plant ‘Sunki Maravilha’ leaves, the gene expression profile of the NCED family genes was not observed. When the soil water content was 0.29–0.28 $\text{m}^3 \text{m}^{-3}$ (control conditions), a small induction of approximately twofold was only observed for

NCED2. These results indicate that the NCED2 and NCED5 genes play an important role in ABA biosynthesis in the roots of the mandarin plant ‘Sunki Maravilha’ when it is subjected to water deficiency stress. The distinct expression profile of the NCED genes in ‘Rangpur’ lime (tolerant genotype) and mandarin plant ‘Sunki Maravilha’ (susceptible genotype)

roots was observed. The difference in the NCED gene expression profiles observed in ‘Rangpur’ lime and mandarin plant ‘Sunki Maravilha’ roots explains the different biosynthetic profiles and ABA accumulation values that were observed in the roots (Fig. 6) as a response to water restriction.

Discussion

The fraction of transpirable soil water (FTSW) varied between the treatments and evaluated species. When the FTSW is evaluated, the amount of water in the soil that is used by the plant for transpiration is assumed to vary based on the field capacity (when transpiration is maximal). When the soil water content becomes limiting and the plant reaches its minimum transpiration, it reaches the limit of 10 % of that transpiration. This model seems to be the most realistic as an indicator of the soil water amount that may be extracted by plants via transpiration. Ray and Sinclair [21] evaluated the FTSW and NTR in *Z. mays* and *Phaseolus vulgaris* grown in pots of different sizes and subjected to water deficiency; the authors observed that the decrease in soil moisture and the evaluation of NTR and FTSW were slightly influenced by the pot sizes. This method is adequate to compare the responses of different genotypes to soil water deficiency.

Most crops experience an FTSW reduction between the initial and final times of water deficiency. Plant physiological changes are initiated in response to the first signs of water deficiency, when the FTSW is between 0.5 and 0.2 [22], promoting stomatal closure and decreased transpiration rates [23].

The different the NTR and FTSW profiles that were observed between the ‘Rangpur’ lime and mandarin ‘Sunki’ plants are related to the different ABA accumulation profiles observed in the leaves and roots under water deficiency stress; a difference in leaf growth and stomatal resistance was simultaneously observed between the genotypes. Although the leaf area growth profiles of the ‘Rangpur’ lime and ‘Sunki Maravilha’ plants subjected to water deficit did not statistically differ from the control, a difference in the growth rate was observed between the genotypes is observed; the ‘Rangpur’ lime growth rate was high, and that of ‘Sunki Maravilha’ tangerine was low (Fig. 2). The lower leaf growth rate of ‘Sunki Maravilha’ may be associated with its greater leaf ABA content. Conversely, the ‘Rangpur’ lime plants displayed a high growth rate even when grown under water restrictions. In citrus plants, this behaviour has been reported as a determining factor for evaluating the transpiration rate [4]. Moya et al. [24, 25] showed that in citrus plants grown under water deficiency, the transpiration rate increased

with respect to the removed leaves and did not alter the transpiration of the whole plant. Therefore, the change in the speed of the absorption of soil water by the roots is associated with the close relationship between leaf area and transpiration rate [26].

During water deficiency, ABA induces stomatal closure [27], which represents one of the initial plant responses to soil water deficiencies and facilitates water retention to promote longer survival times under water stress conditions [28]. The stomatal resistance data indicate that a soil water content of $0.33 \theta\text{-m}^3 \text{m}^{-3}$ for ‘Sunki Maravilha’ and $0.19 \theta\text{-m}^3 \text{m}^{-3}$ for ‘Rangpur’ lime, plants require additional energy input because the soil water content is too low (Fig. 3). ‘Sunki Maravilha’ reached a greater stomatal resistance value (for which the transpiration rate was minimal) when the soil had a larger water content when compared to ‘Rangpur’ lime. Even under these water deficient conditions, ‘Rangpur’ lime were more tolerant to drought than ‘Sunki Maravilha’; the former plant displayed a higher transpiration rate and lower FTSW. This result suggests that ‘Rangpur’ lime is more efficient at acquiring soil water at lower FTSW values when compared to ‘Sunki Maravilha’. A greater ABA accumulation profile difference was observed between ‘Sunki Maravilha’ and ‘Rangpur’ lime under these conditions in the roots and leaves (Fig. 6). Field experiments under water deficit indicated that the combination ‘Valencia’/‘Sunki Maravilha’ presented leaf epinasty and leaf fall, whereas the combination ‘Valencia’/‘Rangpur lime’ maintained leaf turgor under the same water deficit conditions (Supplementary Fig. 1).

Rodríguez-Gamir et al. [4] evaluated the rootstocks of citrus plants (*Cleopatra mandarin* and *Poncirus trifoliata*). Under water deficiency, these rootstocks may actively respond to drying soil by sending signals to the leaves to induce stomatal closure, thereby reducing water loss by transpiration.

Endogenous ABA levels are controlled by the equilibrium between ABA biosynthesis and catabolism; therefore, ABA biosynthesis must be stimulated to induce and maintain stomatal closure during water stress [29, 30]. ABA plays an important role in the initial acclimation response of plants to different environmental stresses and several physiological processes [8, 9]. ABA is transported to the leaves through xylem vessels and ultimately reaches the guard cells, where it can affect transpiration flow. This root-to-leaf transport is initiated by a signal released by genes related to its induction [9]. It is important to consider that several factors may be related to the regulation of ABA levels. The mechanisms that are related to the regulation of ABA levels help to re-adapt the plants to survive under different stress conditions.

Leaf water potential is a thermodynamic property that determines how much water is available to perform cellular

chemical reactions in tissues [31]; when it is measured during the predawn hours, it provides an indirect measure of the soil water potential in which the plants are located. During this time period, evapotranspirative water loss is zero or negligible due to the absence of light and high moisture content (nearly saturated); under these conditions, the soil, plant and atmospheric water contents exist in equilibrium. The more negative the water potential value, the less water is available to perform chemical work, stimulating a series of physiological, morphological and molecular responses, which enables increased water conservation. Water conservation occurs to avoid or mitigate the negative effects of drought, which ultimately culminates in growth inhibition and decreased production [32].

Both genotypes displayed similar leaf water potential profiles (Fig. 5) for all of the studied points. The water content at 0.15–0.14 was significantly different from the control at 20–19 $\text{m}^3 \text{m}^{-3}$. The time that was required for each genotype to reach 36 and 40 days to reach a lower value of water content value for the ‘Rangpur’ lime and ‘Sunki Maravilha’, respectively, should be considered. ‘Rangpur’ lime demonstrated a higher speed in using water; this characteristic confers greater drought tolerance under field conditions (Supplementary Fig. 1). This decreased leaf water potential is consistent with the observed stomatal resistance and NCED gene expression data, indicating an important correlation between physiological and molecular processes in plants subjected to extreme stresses. No water potential difference was observed between the genotypes when the soil water contents ranged from 0.29–0.28 to 0.17–0.16 $\text{m}^3 \text{m}^{-3}$. Although the ‘Rangpur’ lime stomata transiently opened (Fig. 4) due to ABA chemical signalling (Fig. 6).

During water deficiency conditions, the ABA levels in plants increase, leading to at least two stress tolerance mechanisms; the stomata close to limit water loss, and gene expression is altered. In response to dry soil, ABA signals are transported from the roots to the leaves to induce stomatal closure [33]. This process was observed in ‘Rangpur’ lime plants, which contained higher ABA levels in the roots than in the leaves. The roots and leaves showed similar ABA biosynthetic profiles at different time points. When the soil water content was 0.15–0.14 $\text{m}^3 \text{m}^{-3}$, the NCED2, 3 and 5 and CCD4a genes were more highly expressed in both tissues. This result may be correlated with ‘Rangpur’ lime precisely because it contains the most efficient root system. The root ABA content sharply decreased in this genotype when it was grown at 0.17–0.16 $\text{m}^3 \text{m}^{-3}$, and a slight increase was observed at 0.15–0.14 $\text{m}^3 \text{m}^{-3}$. High ABA levels are known to trigger ABA degradation [34–36], which explains the sudden reduction in ABA levels when the soil water content is 0.17–0.16 $\text{m}^3 \text{m}^{-3}$, leading to an

ABA accumulation peak (Fig. 6c, d). Under ideal conditions, the size aerial portion of the plant is proportional to the size its root system. The leaf analysis data (Fig. 2) and high ABA content at 0.20–0.19 $\text{m}^3 \text{m}^{-3}$ (Fig. 6) supports the transpiration rate regulation in the ‘Rangpur’ lime plant. The differences in leaf area, ABA content and gene expression observed between the species may also be related to the higher soil water collection efficiency of the ‘Rangpur’ lime plant.

Several studies have been conducted to elucidate the action of genes that encode the protein functions involved in ABA biosynthesis under abiotic stress conditions, primarily water deficiency stress. Biochemical and genetic studies have shown that the NCED-catalysed cleavage reaction of *cis*-epoxycarotenoid in xanthophyll plays a crucial role in the regulation of ABA biosynthesis in plants subjected to abiotic stress [8, 37]. The NCEDs are carotenoid dioxygenase enzymes (CCDs), which belong to a group that produces specific cleavages in certain double-bonded molecules [38]. Several studies have focused on NCED gene regulation in response to water deficiency. These studies have indicated that the NCED-catalysed cleavage reaction is a key step in the regulation of ABA synthesis when induced by water stress [11, 38].

The current work evaluated the comparative gene expression of the NCED family in the leaves and roots of ‘Rangpur’ lime and mandarin plant ‘Sunki Maravilha’ rootstocks subjected to water deficiency (Supplementary Fig. 1). In this experiment, *NCED1* repression was observed; this result is similar to the results reported by Iuchi et al. [14], who analysed different NCED genes in *A. thaliana* subjected to water deficiency. Although nine NCED/CCD genes have been described in *A. thaliana*, only 5 (AtNCED2, 3, 5, 6 and 9) are involved in ABA production [38, 39]. The remaining four genes differ from NCEDs and have been classified as carotenoid cleavage dioxygenases (CCDs). The role of all of the members of NCEDs family in ABA biosynthesis remain to be investigated because phylogenetic trees have been created based on sequence similarities and the existence of common protein domains [36].

In the leaves of the studied genotypes, *NCED5* was more highly expressed in ‘Rangpur’ lime (Supplementary Fig. 1b) when the soil water content was 0.15–0.14 $\text{m}^3 \text{m}^{-3}$. It is noteworthy that, with respect to the physiological parameters evaluated in this work, when the soil water content was 0.15–0.14 $\text{m}^3 \text{m}^{-3}$ and the plants were subjected to water deficiency, the largest changes in gene expression were observed. With respect to the molecular data, this time period correlated with the activation of defence mechanisms, which conferred a higher resistance to water deficiency. Therefore, in ‘Rangpur’ lime leaves, the *NCED5* gene can be considered to be responsible for the mechanisms conferring drought tolerance and initial ABA biosynthesis. The induction of

NCED genes by water stress has also been reported for *Z. mays*, *Lycopersicon esculentum*, *A. thaliana* and *P. vulgaris* [11, 40, 41].

Although ABA progressively accumulated in the roots and leaves of the mandarin plant ‘Sunki Maravilha’ when the soil water content decreased to 0.20–0.19 and 0.17–0.16 m³ m⁻³, no transcriptional increase was observed in any of the NCED genes. When roots of the Cleopatra rootstock were subjected to severe water deficiency stress for 24 h [6], NCED3 transcription increased by approximately 186-fold during the first 3 h; this increase was followed by a progressive decrease of approximately 90-fold during the period from 3 to 12 h, and this level was stable until the roots were rehydrated despite the progressive increase in ABA accumulation that occurred from the initiation of water deficiency until rehydration. These data support the hypothesis that the time at which leaves and roots are collected from plants grown in soil water contents of 0.20–0.19 and 0.17–0.16 m³ m⁻³ show induced expression of the evaluated genes. A similar result was found for ‘Rangpur’ lime.

When NCED gene expression was evaluated in the roots, *NCED2*, *NCED3* and *CCD4a* (Fig. 7a) were highly expressed in ‘Rangpur’ lime, and *NCED2*, *NCED5* and *CCD4a* were most highly expressed in ‘Sunki Maravilha’ (Fig. 7c). The root expression profile of the NCEDs/CCD genes confirmed the hypothesis that the perception and induction of defence signals in response to water deficiency is initiated in the roots. Several studies have shown that under experimental conditions in which the root system was subjected to water deficiency and the leaf water state was not altered, the signals produced in the roots induced stomatal closure in the leaves [42, 43]. Stomatal closure has been related to increased ABA levels in the xylem, suggesting that the ABA signals originate in the root and are transported to the aerial portion, thus regulating stomatal opening in response to water deficiency [44]. Another important observation is that the NCED3 gene was only expressed only in ‘Rangpur’ lime; this result may be related to specific drought tolerance mechanisms and ABA synthesis profiles in this cultivar. A previous study [6] suggested that the NCED3 gene was more highly expressed in the roots of citrus plants in response to water deficiency and increased ABA concentrations. The Arabidopsis gene *AtNCED5* and its citrus orthologs *CcNCED5* and *CsNCED* [6] are expressed in many non-green, aerial tissues, such as flowers, seeds, siliques and yellow developing anthers, suggesting that *AtNCED5* expression may be related to tissues with a low ratio of chlorophyll/carotenoids. However, it was induced in the leaf under water deficit conditions [14].

The ABA accumulation profile and expression of NCED genes may affect leaf water potential, allowing ‘Rangpur’

lime and the mandarin plant ‘Sunki’ to adapt to water deficiency stress. ‘Rangpur’ lime is a vigorous rootstock, and its leaf growth rate remains unchanged even when water is unavailable. ABA signals sent from the roots to the aerial portions and other chemical signals may increase stomatal resistance, thereby decreasing transpiration. Therefore, ‘Rangpur’ lime uses soil water much more rapidly than the mandarin plant ‘Sunki’, which required more than 5 days to reduce the soil water content (0.15–0.14 m³ m⁻³) when compared to ‘Rangpur’ lime.

Conversely, the mandarin plant ‘Sunki’ drought adapts more uniformly between the root and aerial portion. The ABA levels increased in the roots and leaves as the water restriction increased (Fig. 6). When the soil water content reached 0.15–0.14 m³ m⁻³ (5 days after ‘Rangpur’ lime), the leaves of the mandarin plant ‘Sunki’ became solely responsible for ABA accumulation. The decreased transpiration rate by stomatal closure promotes increased signaling of ABA and other possible chemical compounds; this process limits leaf growth and decreases water flow from the roots to the aerial tissues. Thus, the water requirement for vital processes decreases in the mandarin plant ‘Sunki’, allowing it to adapt to reduced soil water availability. Therefore, the response of the mandarin plant ‘Sunki’ is based on the balance of the response between the roots and the aerial tissues with respect to the water restriction. The mandarin plant ‘Sunki’, in combination with a variety of tree crowns displaying similar characteristics, which respond to water deficiency stress by promoting ABA accumulation in the leaves, confers drought tolerance under field conditions.

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