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Assessing the regulation of leaf redox status under water stress conditions in *Arabidopsis thaliana*

Col-0 ecotype (wild-type and *vtc-2*), expressing mitochondrial and cytosolic roGFP1

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Keywords: *Arabidopsis thaliana*, ascorbate, Col-0, cytosolic roGFP1, glutathione, leaf redox status, mitochondrial roGFP1, recovery, *vtc2*, water stress

Abbreviations: AsA, ascorbate; c-roGFP, cytosolic ro-GFP; DHA, dehydroascorbate; DW, dry weight; FW, fresh weight; GSH, glutathione; GSSG, glutathione disulfide; m-roGFP, mitochondrial roGFP; M, mature; roGFP1, redox sensing green fluorescent protein; *vtc2*, ascorbate deficient mutant; WS, water stressed plants; WW, well watered plants; Y, young

Using *Arabidopsis* plants Col-0 and *vtc2* transformed with a redox sensitive green fluorescent protein, (c-roGFP) and (m-roGFP), we investigated the effects of a progressive water stress and re-watering on the redox status of the cytosol and the mitochondria. Our results establish that water stress affects redox status differently in these two compartments, depending on phenotype and leaf age, furthermore we conclude that ascorbate plays a pivotal role in mediating redox status homeostasis and that Col-0 *Arabidopsis* subjected to water stress increase the synthesis of ascorbate suggesting that ascorbate may play a role in buffering changes in redox status in the mitochondria and the cytosol, with the presumed buffering capacity of ascorbate being more noticeable in young compared with mature leaves. Re-watering of water-stressed plants was paralleled by a return of both the redox status and ascorbate to the levels of well-watered plants. In contrast to the effects of water stress on ascorbate levels, there were no significant changes in the levels of glutathione, thereby suggesting that the regeneration and increase in ascorbate in water-stressed plants may occur by other processes in addition to the regeneration of ascorbate via the glutathione. Under water stress in *vtc2* lines it was observed stronger differences in redox status in relation to leaf age, than due to water stress conditions compared with Col-0 plants. In the *vtc2* an increase in DHA was observed in water-stressed plants. Furthermore, this work confirms the accuracy and sensitivity of the roGFP1 biosensor as a reporter for variations in water stress-associated changes in redox potentials.

Introduction

Maintenance of redox homeostasis is an important mechanism by which plants cope with stress. Thus, knowledge of the location of where redox changes occur, and their kinetics and magnitude, is crucial to understanding the responses of plants to environmental stress. The expression of reduction-oxidation green fluorescent proteins (roGFP1) in plants has become a useful tool as it provides in vivo direct measurements of redox status over time and in different intracellular locations. Using this technique/ approach, it has been established that water deficit can induce redox changes in the plant cell cytosolic matrix, external to the

organelles.¹³ However, because organelles, especially the mitochondria, are sensitive to redox perturbations,²⁹ we concluded that measuring the redox status of the mitochondria, might lead to a deeper understanding of the effects of drought stress.

Redox systems show a high plasticity, with redox homeostasis in plants regulated primarily by antioxidants, such as ascorbate and glutathione,^{9,27} which may act, or not, in a compensatory manner.¹⁹ In the ascorbate-glutathione pathway, reduced glutathione (GSH) regenerates ascorbate (AsA), by reducing dehydroascorbate (DHA), either chemically or via dehydroascorbate reductase enzymes (DHRA). However, AsA regeneration may be independent of GSH, since the DHRA pathway is only one of

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several routes for GSH oxidation. Plant cells are able to maintain very high cytoplasmic AsA:DHA ratios, presumably because of efficient GSH-independent pathways for AsA regeneration.⁹ Moreover, in the intracellular environment the overall abundance of reduced ascorbate may be more important in terms of regulation, than the AsA:DHA ratio. Under non-stressful conditions (e.g., well-watered plants) ascorbate and glutathione in young leaves are highly reduced, but they shift toward the oxidized forms under stress,^{9,22} altering the redox status and therefore the biological activities of target proteins. While changes in AsA:DHA ratio are frequently considered to be a redox status indicator, presently there are no clear mechanisms that directly link the AsA:DHA ratio to altered redox status and to the biological activities of target proteins. However, previous work using c-roGFP1 suggests that cytosolic redox status and AsA and DHA content are tightly regulated and linked.¹³

Arabidopsis mutants, such as the ascorbate-deficient mutant, *vtc2*, have provided unequivocal evidence that ascorbate is a multifunctional metabolite that is important in redox homeostasis and in signaling, as well as in development and defense reactions.^{7-9,26} Indeed, ascorbate deficiency slows the growth of the shoot and the root,²³ and it has been reported that mutants deficient in ascorbate (e.g., *vtc2*) attain less biomass.²⁵ But surprisingly, *vtc2* mutants do not show oxidative stress and, the lack of AsA is not compensated by changes in the other major antioxidants, except by ascorbate peroxidase (APX).⁵

Whereas stresses such as drought may influence the amounts and the redox status of both ascorbate and glutathione, it is clear that other factors, such as plant age, may also be related to the forms and total content of both ascorbate and glutathione.^{1,28} Therefore, any assessment of the effects of drought stress on leaf redox status must also include a consideration of leaf/plant age.

Another consideration is that most studies of the influence of stress on redox status and antioxidants are performed in plants subjected to a short, intensive period of stress, without further assessing how the redox status adjusts following re-watering and recuperation of the plant, which would be similar to conditions plants encounter in the field. During the stress it is hypothesized that the plant needs to maintain tight control of both redox homeostasis during water stress and the reversible redox regulation of proteins by dithiol-disulfide exchange, as well as after re-watering.³¹

In the current work we explore the role(s) of ascorbate in water-stressed plants and subsequent recovery, with a focus on redox status. Using the *Arabidopsis* mutant (*vtc2*) impaired in its capacity to synthesize ascorbic acid, we investigate the possibility of ascorbate mediating crosstalk between water stress and recovery. Additionally, because of an interaction between ascorbate and glutathione cycles in regulating overall redox status,⁹ we also assess biochemically changes in the glutathione (reduced/oxidized) pool, using these same mutants. Because earlier work has shown that *vtc1* mutants do not present symptoms of oxidative stress under non-stressed growth conditions, we consider the possibility that there are changes in glutathione levels which compensate for alterations in ascorbate levels, thereby potentially

enhancing in ascorbate deficient mutants redox signaling through glutathione-dependent pathways.⁹

Because redox status can differ in different cellular compartments, we also monitored the redox status in both the cytosol and mitochondria during water stress and subsequent re-watering. For this effort, we monitored in real time, dynamic changes in cytosol and mitochondria redox status in leaves of both wild-type and *vtc2* mutants transformed with one of the two forms of roGFP1; targeted to the cytosol (c-roGFP1), or targeted to mitochondria (m-roGFP1). The time course of leaf water relations, plant biomass, ascorbate levels and redox status and cytosolic as mitochondrial redox status were monitored. Also leaf ontogeny has been considered. From this work we have established that roGFP1 is sufficiently sensitive to visualize the redox dynamics at the physiological level in both the cytosol and mitochondria in plants subjected to water stress followed by recovery. Moreover, information on the possible crosstalk between subcellular redox status, coupled with measurements of the ascorbate and glutathione pools, in relation to leaf ontogeny, offer insights of the complexity of plant responses to water stress.

Results

Relative water content of WW *Arabidopsis* (wild-type and *vtc2* expressing ro-GFP1 in either the cytosol or mitochondria) was maintained between 80% and 92% during the duration of the experiment. In contrast, under WS treatment significant differences started to appear in all lines after 10 d of water depletion. Re-watering was performed on day 18 and measurements were made 6 h after and 6 d after the re-watering (on days 19 and 24 of the experiment, respectively) and by then the water stressed plants leaf RWC had recovered to WW plant values, around 90%, indicating that plants were subjected to a mild water stress (Fig. 1).

Measurements of soil water content (SWC) showed differences from the very beginning of the experiment between WW and WS treatments. Soil water content of the WW lines was maintained between 5 and 6 g H₂O g⁻¹ DW. In contrast, WS pots experienced a 50% loss before day 10 and values below 1 g H₂O g⁻¹ DW between days 14 and 18 were observed. Day 19 (immediately after the beginning of recovery) and day 24 (6 d after beginning recovery) showed that the soil was rapidly rehydrated, but without reaching WW values (Fig. 2).

Irrespective of the type of transformation (i.e., with either c-roGFP1 or m-roGFP1) no significant differences were observed in rosette biomass, when making comparisons within individual lines (Col-0 and *vtc2*). This observation held true for all sampling time points. Thus, because of this consistency, only one plot per mutant and water treatment is shown. Rosette biomass increases progressively through the experiment in WW plants, but to a lesser extent in *vtc2* than in Col-0 and, by the end of the experiment rosette biomass of *vtc2* (Fig. 3B) than in Col-0 (Fig. 3A) and, by the end of the experiment the rosette biomass of *vtc2* mutants was ca. 28% lower than in Col-0. A decrease in rosette biomass was observed in WS plants because of the lower RWC and was more noticeable by the end of experiment when a

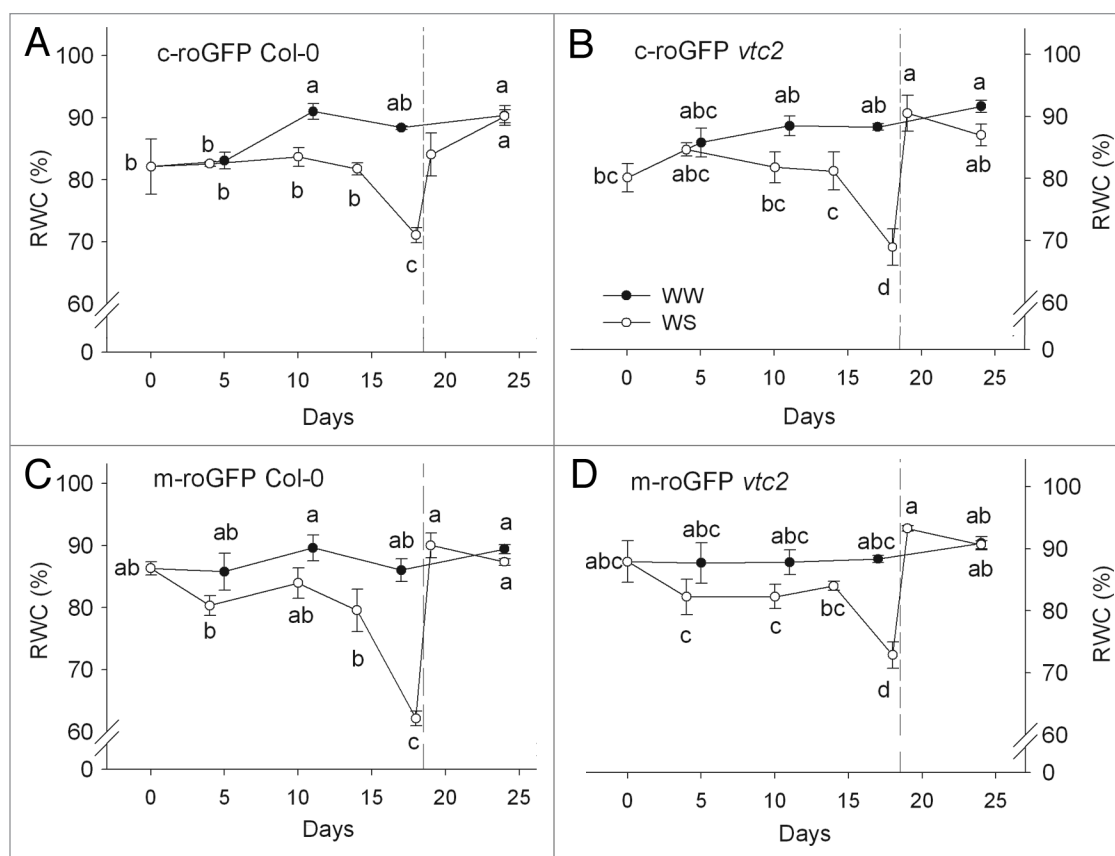


Figure 1. Relative water content (%) of *Arabidopsis thaliana* Col-0 (**A and C**) and *vtc2* (**B and D**) plants transformed with c-roGFP (**A and B**) and m-roGFP (**C and D**) through the time course of the experiment in well watered (WW) and water-stressed (WS) treatments. Vertical dashed line indicates the re-watering of water-stressed plants. Values with different letters indicate significant differences at $p < 0.05\%$ according to the Duncan multiple range test. Data are means $\pm$ SE, $n = 5$.

difference of ca. 49% was observed between WW and WS Col-0 plants and of ca. 40% in *vtc2*.

Redox status of cytosol and mitochondria is related to leaf ontogeny and water stress. Monitoring of cytosol redox potential in wild-type plants expressing c-roGFP1 (Fig. 4A) revealed that on day 14 of water deprivation the redox potential increases, becoming more oxidized in both young and mature WS leaves, reaching its most oxidized level on the maximum stress day (day 18), when WS plants presented values of RWC ca. 60%, a 15% lower than WW plants. The mature (M) and young (Y) leaves clearly have different ranges of redox potential: in the cytosol of WW plants values were found between -307 and -303 mV in M leaves and below -310 mV (less oxidized) in Y leaves. Under maximum WS, wild-type M leaf cytosol reached a maximum of -295 mV, while Y leaves reached only -299 mV. With re-watering and recovery of WS plants, the redox potential at day 24 decreased back to -306 and -312 mV (a more reduced status) in M and Y leaves, respectively. Subsequently, mild water stress induces a reversible oxidative stress. Moreover, the redox potentials of WW plants show a tendency to become less negative (more oxidized), through the course of the experiment and showed a relationship between leaf age and redox potential.

For *vtc2* plants values for the cytosol redox status (Fig. 4B) in WW plants showed similar trends as observed in the wild-type,

although, unexpectedly under WS, redox values appear lower (more negative) and thus less oxidized than in the wild-type cytosol and also differences between leaf typology were less noticeable. Mutant *vtc2* plants reached a maximum potential cytosolic redox value of -301 mV in M leaves cytosol on day 18 of WS and a maximum of -307 mV in the cytosol of Y leaves. With re-watering, recovery of the redox potential of the WS plants was similar to that of WW wild-type plants. This suggests that a ascorbate levels in the *vtc2* plants, though 80% less than wild-type, may still be adequate to buffer WS-induced redox changes. Alternatively, another mechanism, not related with the levels of ascorbate, may function to protect *vtc2* mutants from intensive changes in oxidative redox status.

Mitochondria redox potential was also monitored in wild-type and *vtc2* plants (Fig. 5). For WW Col-0 plants the mitochondrial redox status was more negative (more reduced) than in the cytosol (Fig. 5A) for both types of leaves. Under WW conditions the mitochondrial redox potential of wild-type mature leaves became more oxidized, from -360 mV to -351 mV with plant age, (Fig. 5A). However, the effects of age along the experiment are not observed in young leaves, which remain more reduced compared with mature leaves, showing values of ca. -358 mV.

For the *vtc2* mutant (Fig. 5B) similar values and trends were also observed; in WW mature leaves mitochondrial redox

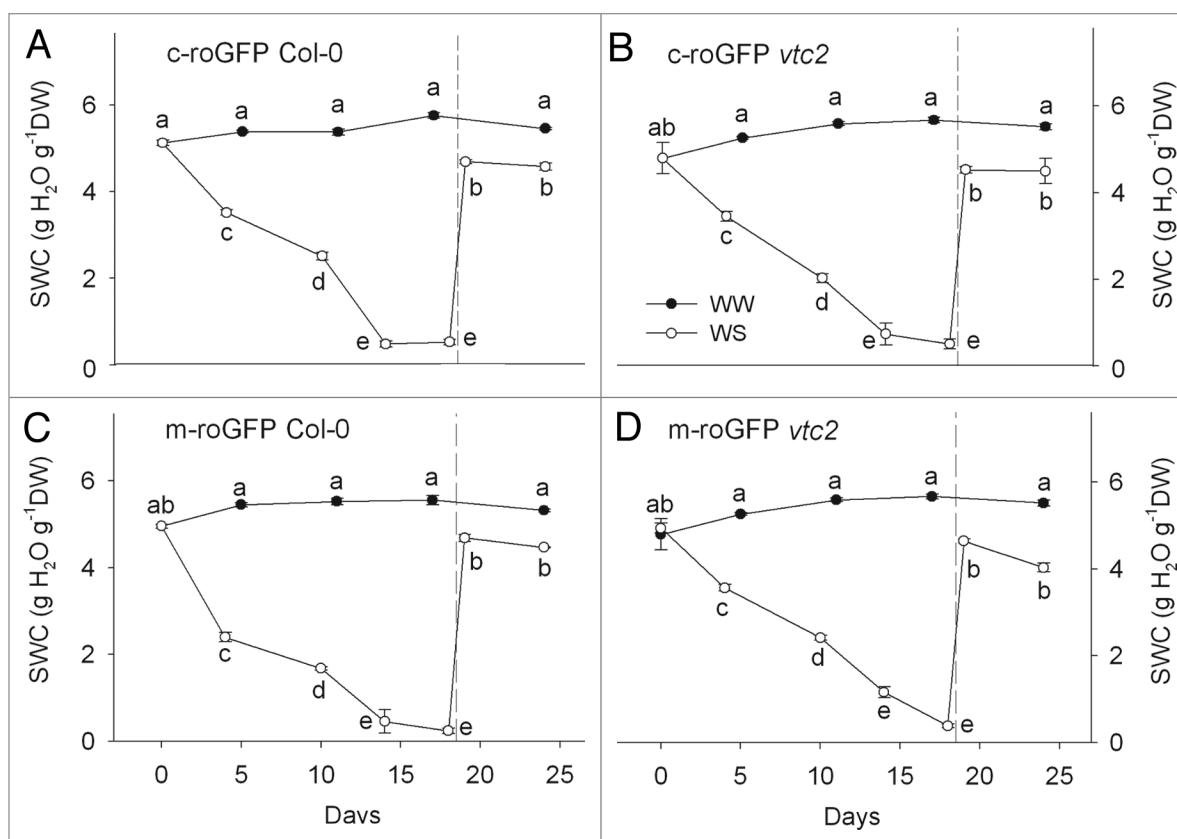


Figure 2. Soil water content of pots containing *Arabidopsis thaliana* Col-0 (**A** and **C**) and *vtc2* (**B** and **D**) plants transformed with c-roGFP (**A** and **B**) and m-roGFP (**C** and **D**) through the time course of the experiment in well watered (WW) and water stressed (WS) treatments. Vertical dashed line indicates the re-watering of water-stressed plants. Values with different letters indicate significant differences at $p < 0.05\%$ according to the Duncan multiple range test. Data are means $\pm$ SE, $n = 5$.

potential increases (becomes more oxidized) with plant age, from a most reduced value of -359 mV on day 4, to a more oxidized values of -352 mV at the end of the experiment. Under WS treatment the redox potentials for mitochondria in *vtc2* plants showed remarkable differences between M and Y leaves; while redox potential increases (becomes more positive) with WS in M leaves (reaching a maximum of -349 mV on day 18), in Y leaves remains constant during the entire time course of stress (ca. -360 mV). Since these trends are the same for both Col-0 plants and *vtc2*, differences in redox potentials are therefore related to leaf age. Six days after beginning watering recovery the redox potential of WS plants recovered to more reduced values of -354 and -359 mV for old and young leaves, respectively.

Redox status, ascorbate and glutathione. Total leaf ascorbate content (AsA+DHA) was determined during time course of water deprivation and recovery in M and Y leaves of wild-type and *vtc2* transformed lines. In order to ascertain whether expression of roGFP1 affected total ascorbate content, ascorbate levels were measured and compared in roGFP1-transformed and non-transformed wild-type and *vtc2* plants under both WW and WS conditions. No differences were noted in ascorbate content so we conclude that being transformed with roGFP1 does not affect ascorbate levels.

In WW Col-0 plants total ascorbate leaf content ranged from 1 to $1.9 \mu\text{mol g}^{-1}$ FW in M leaves and from 1.7 – $2.8 \mu\text{mol g}^{-1}$ FW in Y leaves (Fig. 6A). Under WS treatment total ascorbate increases in comparison with the WW treatment, but with M leaves showing significant lower ascorbate content than Y leaves (Fig. 6A and B). DHA increases successively, both on day 14 and 17 showing a significant increase prior to water recovery and significantly decreases during WS recovery (Fig. 6B).

Total ascorbate levels in *vtc2* mutants were about 80% lower than in Col-0 plants (Fig. 6C). Young leaves had significantly higher levels of ascorbate than mature leaves (about 50% more) and higher DHA levels during WS that significantly decrease after re-watering (Fig. 6D).

Leaf glutathione was also determined from the same extract used for measurements of ascorbate (Fig. 7). No significant differences were observed in the Col-0 ecotype between WW and WS treatments. However, glutathione content in Y leaves (ranging from 0.37 – $0.57 \mu\text{mol g}^{-1}$ FW) was higher (a 2–8% increase) compared with M leaves (ranging from 0.26 – $0.37 \mu\text{mol g}^{-1}$ FW) during the time course in both treatments (Fig. 7A and B). During recovery the fast answer of Y leaves was shown by a significant increase after 6 h (19 d) and back to previous values after 6 d (19 d) (Fig. 7B).

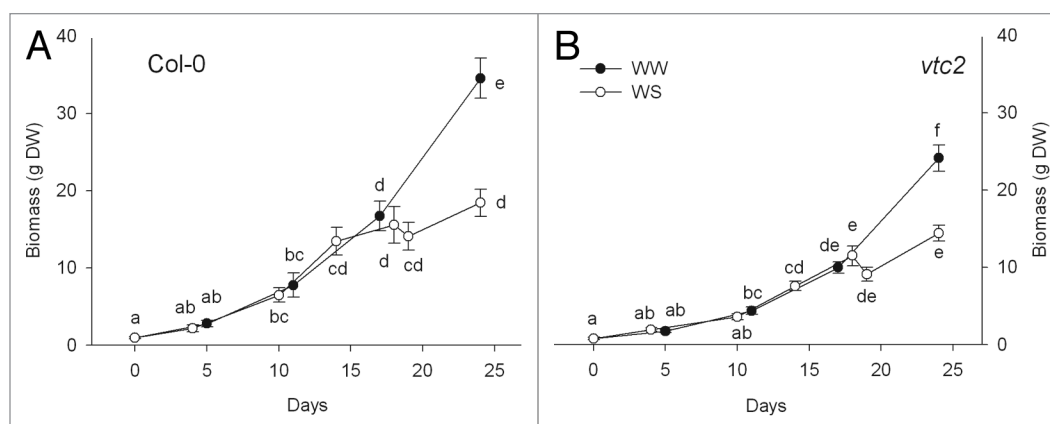


Figure 3. Biomass (g) evolution of the Col-0 (A) and *vtc2* (B) through the time course of the experiment in well watered (WW) and water-stressed (WS) treatments. Values with different letters indicate significant differences at $p < 0.05$ according to the Duncan multiple range test. Data are means $\pm$ SE, $n = 5$.

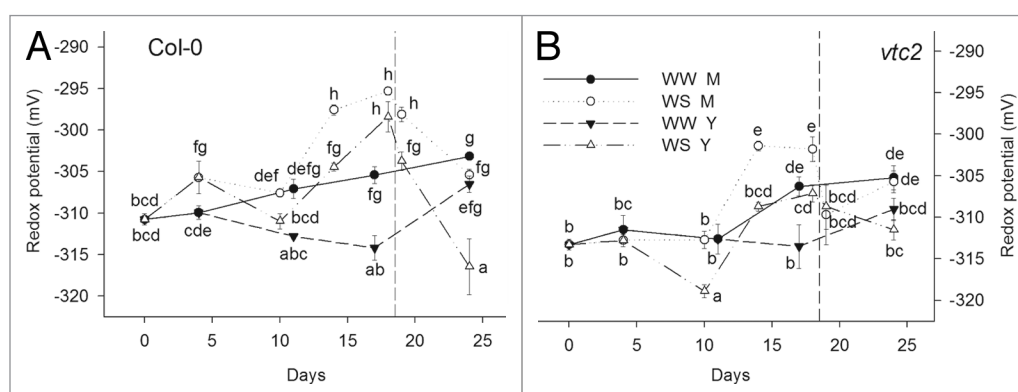


Figure 4. Changes in redox potential of cytosol (c-roGFP) transformed *Arabidopsis thaliana* Col-0 (A) and *vtc2* (B) of mature (M) and young (Y) leaves through the time course of the experiment in well watered (WW) and water-stressed (WS) treatments. Vertical dashed line indicates the re-watering of water-stressed plants. Values with different letters indicate significant differences at $p < 0.05$ according to the Duncan multiple range test. Data are means $\pm$ SE, $n = 5$.

The levels of total glutathione in *vtc2* mutants tend to increase through the experiment in WW plants (about 40% at day 19 above day 0) and also total glutathione content increases (4–10%) in young compared with mature leaves (Fig. 7C and D). Through the duration of the experiment, and up until after 6 h re-watering, glutathione increases in both Y and M leaves of water-stressed plants, reaching values about 40% higher than day 0. One week after re-watering and recovery (day 24) the levels of total glutathione decrease to values of $0.35 \mu\text{mol g}^{-1}$ FW in both M and Y leaves, similar to values observed at the beginning of the experiment. The peak of glutathione after 6 h of re-watering was not only significant in Y leaves but also in M (by almost 2-fold higher than maximum stress values).

Discussion

Here, we report a comparative study of the effects of a progressive water stress and recovery, coupled with simultaneous measurements of leaf ontogeny, ascorbate and glutathione content,

on the redox status of two cellular compartments (mitochondria and the cytosol) in wild-type and *vtc2* mutant *Arabidopsis*. In previous work,¹³ using c-roGFP1, we investigated the effects of water stress and recovery on cytosolic redox status in wild-type *Arabidopsis*. Because that earlier work showed significant changes in ascorbate (in both oxidized and reduced forms) with the onset of water stress, we wanted to explore further a presumed association of ascorbate with water stress. This prompted us to examine ascorbate biochemistry in an ascorbate-deficient mutant (*vtc2*) subjected to water stress. Moreover, because the ascorbate and glutathione cycles are so intimately coupled in the control of plant redox status,⁹ we also concluded that measurements of glutathione content could be informative in understanding homeostasis of cellular redox status in plants subjected to water stress. Finally, because GSH exerts a major influence on cellular redox status by affecting the redox balance of mitochondria,^{16,24} we also explored and compared the redox status of mitochondria (using a roGFP1 targeted to mitochondria) with the redox status of the cytosol in water-stressed plants. This report is therefore the first

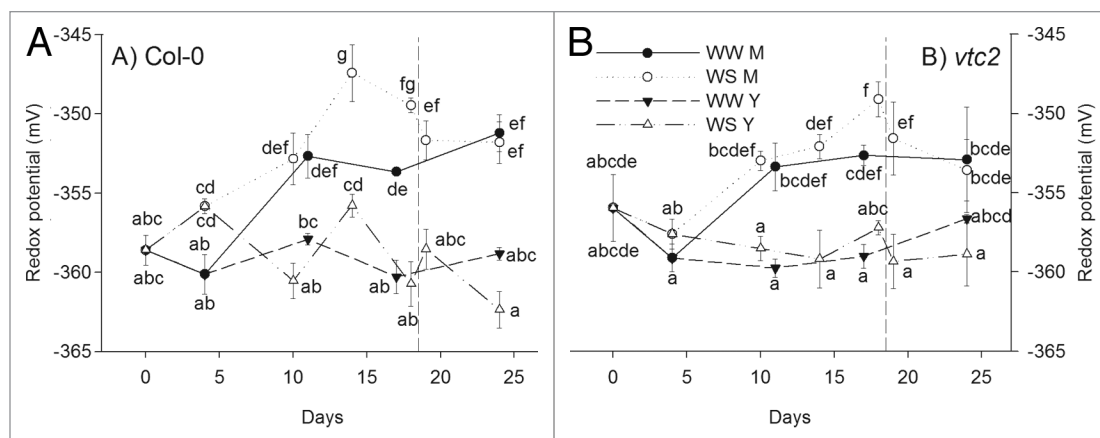


Figure 5. Changes in redox potential of mitochondria (m-roGFP) transformed *Arabidopsis thaliana* Col-0 (A) and *vtc2* (B) of mature (M) and young (Y) leaves through the water stress treatment in well watered (WW) and water-stressed (WS) treatments. Vertical dashed line indicates the re-watering of water-stressed plants. Values with different letters indicate significant differences at $p < 0.05\%$ according to the Duncan multiple range test. Data are means $\pm$ SE, $n = 5$.

to investigate, in real time, parallel changes in the redox status of both the cytosol and mitochondria in *Arabidopsis* lines subjected to a progressive water stress, followed by recovery. Additionally, from this effort we confirmed both the accuracy and sensitivity of the roGFP1 biosensor as a reporter for variations in water-stress associated changes in redox potentials. Finally, we consider how these findings shed light on the regulation of the subcellular homeostasis of redox status.

Water stress affects plant biomass. Water stress results in a reduction in biomass that becomes significantly different from WW plants in day 10 for the Col-0 but only after water recovery for *vtc2* line. During the water withholding, the total biomass for the *vtc2* line was identical for both the WW and WS plants. This suggests that a reduction in ascorbate levels in the *vtc2* mutant did not impair the ability of this line to respond to WS, at least up to day 18, following the same growth ratio as WW plants. Moreover, at day 24 the biomass for WS *vtc2* line is only 16% lower than for WS Col-0 and, again supporting the view that the reduced levels of ascorbate in *vtc2* possibly are not linked to the reduction in biomass due to WS. However, the fact that the biomass of the WW *vtc2* plants is significantly less at day 24 compared with the biomass of the WW Col-0 plants, suggests that a reduced level of ascorbate in WW *vtc2* plants is not without consequence and thereby points to a role for ascorbate in plant growth under non-stressed conditions.³⁴

Effects of water stress on redox status differ depending on phenotype, leaf age and cellular compartment. Water stress affects the redox status differently in the Col-0 vs. the *vtc2* line. In the Col-0 line the most oxidized status of the mitochondria is reached 14 d after withholding water for both Y and M leaves, whereas in the *vtc2* mutant the mitochondria compartment was at its most oxidized just before re-watering (18 d after beginning to withhold water). On the other hand, cytosol redox values for the Col-0 line were the most oxidized just before re-watering and attained values of redox potential less negative (more oxidized) than mitochondria. Our results show that mitochondria on both Col-0 and *vtc2* lines are better buffered than are the cytosol.

These results are in accordance with Jiang et al.¹² who showed that mitochondria are much better than cytosol in buffering changes in redox potential.

The redox status of the mitochondria compartment also appears related to leaf age, with younger leaves of both the Col-0 and *vtc2* lines consistently exhibiting a more reduced redox status than older, more mature leaves. But with regard to the redox status of the cytosol compartment, its relationship between leaf age is less pronounced. From this work we conclude that the mitochondrial and cytosolic compartments respond differently to WS, with regard to redox status.

Moreover, because no significant differences were seen in mitochondria redox potentials in young leaves between WW or WS, this again points to the high buffering capacity of mitochondria, especially in young leaves.

It is known that mitochondria are highly resistant to abiotic stress³ which our work now suggests could be related to their high capacity of redox homeostasis. Additionally, because mitochondria can remain intact until the last stages of senescence¹⁵ and this could explain our observation in WS mature leaves mitochondria remain more reduced than the cytosol.

In young leaves of WW plants (Col-0 and *vtc2*), there are no significant differences in redox status for the duration of the experiment at the mitochondria. Because mature leaves of WW plants tend to be more oxidized throughout the experiment, this increase in ROS could underlie the leaf senescence process.²⁰ Next, we consider changes in redox status in relation to variations in the levels of ascorbate and glutathione.

WS-induced changes in ascorbate and glutathione. Both the oxidized and reduced forms, and total amounts, of ascorbate and glutathione change in response to WS. Most pronounced are changes in AsA and DHA in young leaves, and to a lesser extent, in mature leaves, after 14 d withholding water, with (total ascorbate peaking at day 18). Even after re-watering, AsA levels continue to rise (on day 19), though total measured ascorbate (oxidized and reduced forms) is somewhat less than on day 18. To some degree this same pattern is

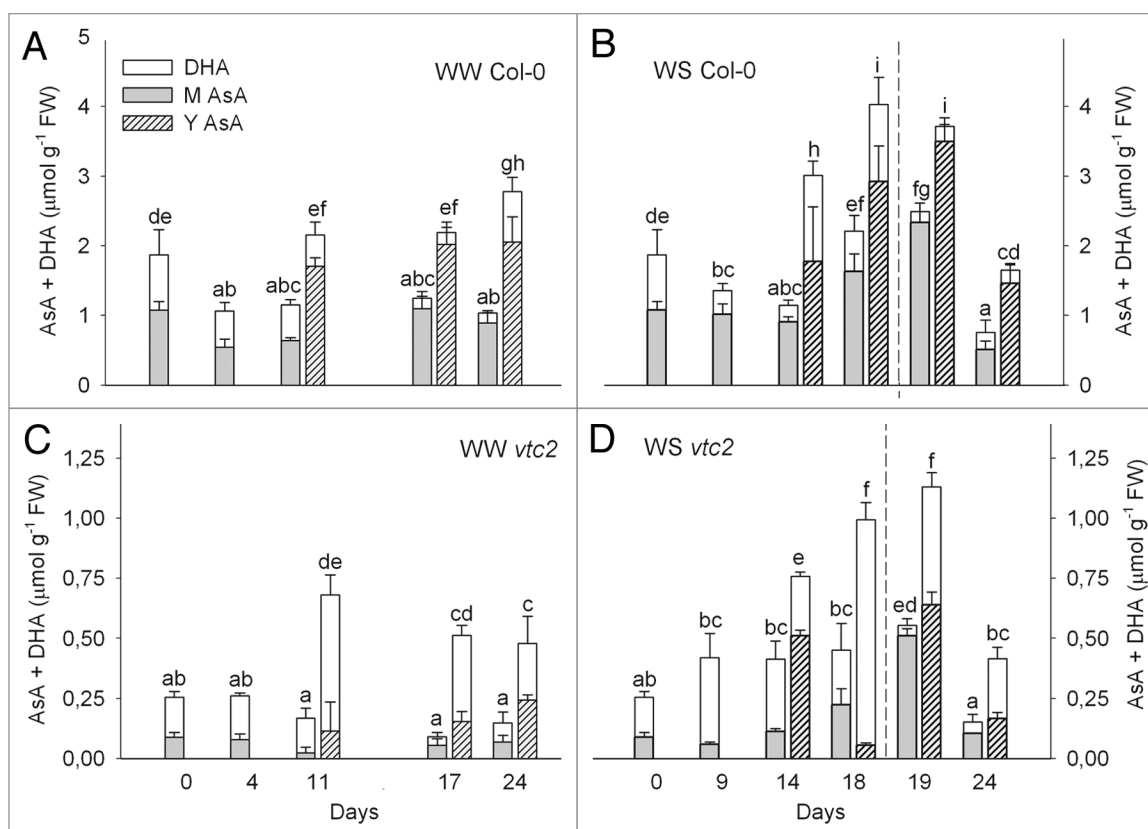


Figure 6. Changes in AA and DHA ($\mu\text{mol g}^{-1}$ FW) in mature (M) and young (Y) leaves from Col-0 (A and B) and *vtc2* mutants (C and D) through the time course of the experiment in well watered (WW) and water-stressed (WS) treatments. Vertical dashed line indicates the re-watering of water-stressed plants. Values with different letters indicate significant differences at $p < 0.05$ according to the Duncan multiple range test. Data are means $\pm$ SE, $n = 5$. As no significant differences in Ascorbate content appeared between c-roGFP and m-roGFP transformed plants, means include the average values of both. Different letters indicate significant differences at $p < 0.05$ Data are means $\pm$ SE, $n = 3$.

observed in the WS *vtc2* line with a peak at day 17, when, however, in young leaves reduced ascorbate is undetectable, but still with relatively high levels of DHA. This suggests that the prolonged WS efficiently causes oxidation of the reduced form (AsA) to DHA and/or negatively affects the regeneration of AsA. For WS *vtc2* plants, the ascorbate buffering could still have been just enough to buffer WS-induced redox changes. Alternatively, another mechanism, not related with the levels of ascorbate, may function to protect *vtc2* mutants from extensive changes in oxidative redox status. With re-watering the level of AsA increases (DHA levels remain unchanged compared with day 17), suggesting that WS may also affect AsA synthesis. In summary, while there are many subtle differences in AsA/DHA levels induced by WS, and linked to leaf age, the most pronounced effects on ascorbate levels under WS were observed in young leaves. In parallel, young leaves showed a more reduced redox potential than mature leaves. Moreover, the last steps of ascorbate biosynthesis are located in mitochondrial inner membrane^{2,18,30} and thus the increase in ascorbate biosynthesis in Col-0 Y leaves would explain the low redox potential in mitochondria throughout WS treatment.

Unlike the situation for ascorbate, WS results in less significant, if any, changes in glutathione (GSH and GSSG). In both the Col-0 and the *vtc2* lines GSH and GSSG amounts remain

relatively unchanged through day 18 and only increase after re-watering. This suggests that these plants do not rely on changes in GSH/GSSG to buffer WS, at least through day 18, and also that because re-watering results in an increase in total glutathione (notably in the young *vtc2* leaves), that glutathione synthesis may be impaired by prolonged water stress. Additionally, even in the ascorbate-deficient mutant, glutathione levels remain more or less unchanged in WS plants, pointing to the fact that no increase glutathione occurs to compensate for a reduction in ascorbate. In addition, though mitochondria appear to be heavily buffered by glutathione,^{17,35} the increasingly oxidized redox status, with the prolongation of WS, can be explained as a consequence of no change in GSH/GSSG levels. On the other hand, because changes in the redox status of the mitochondria (which start to become more reduced after day 14) parallel increases in ascorbate, this suggests that in the mitochondria ascorbate is the major redox buffering species. This view is supported by essentially no change in mitochondrial redox status in the ascorbate-deficient mutant.

The results observed in AsA levels in the *Arabidopsis* Col-0 line plants subjected to water stress and cultivated in a growth chamber cannot now be extrapolated in its totality to plants subjected to drought and growing under field conditions. Nevertheless, our results suggest that even in plants growing in natural conditions

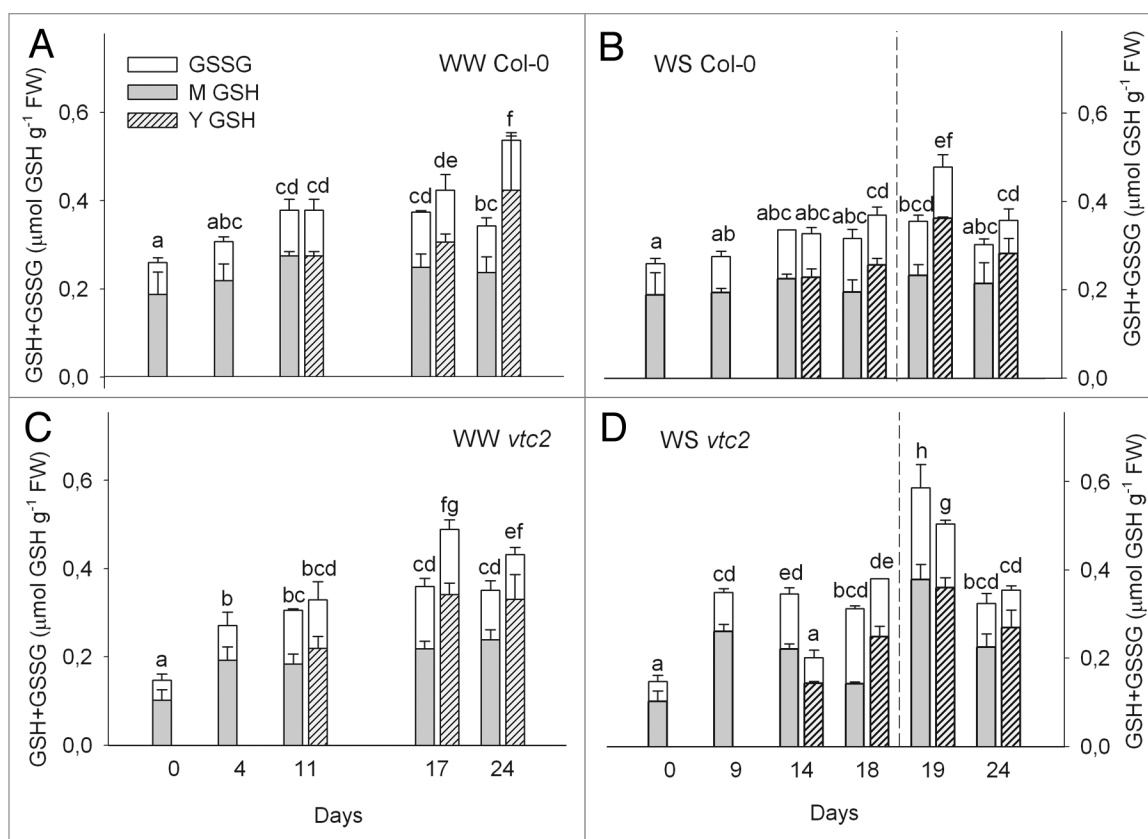


Figure 7. Changes in reduced and oxidized glutathione (GSH+GSSG) in mature (M) and young (Y) leaves from Col-0 (**A** and **B**) and *vtc2* (**C** and **D**) mutants through the time course of the experiment in well watered (WW) and water-stressed (WS) treatments. Vertical dashed line indicates the re-watering of water-stressed plants. Values with different letters indicate significant differences at $p < 0.05$ according to the Duncan multiple range test. Data are means $\pm$ SE, $n = 5$. As no significant differences in glutathione content appeared between c-roGFP and m-roGFP transformed plants, means include the average values of both. Different letters indicate significant differences at $p < 0.05$. Data are means $\pm$ SE, $n = 3$.

and subjected to a progressive water stress, that variations in the endogenous concentrations of AsA will likely be useful as stress markers, since plants resistant to drought generally show an increase in AsA levels.¹⁴

In summary, the results of measurements of ascorbate and glutathione accord well with the roGFP data. Most notable is the fact that the redox status of cytosol of WS Col-0 plants continues to become more oxidized with prolongation of WS, whereas the redox status of the mitochondria starts to become more reduced at about day 14. We therefore conclude that *Arabidopsis* responds to WS by increasing the synthesis of ascorbate, which primarily buffers changes in the mitochondria. Second, WS appears not to result in a significant increase in glutathione synthesis (or in the amounts of GSH or GSSG). Thus, if ascorbate is the major redox-buffering species in water-stressed plants, and if its reduced form normally involves the glutathione cycle, then the increase in AsA/DHA in WS plants may occur via other reductants, in addition to GSH.

Material and Methods

Plant material and water stress treatment. Seeds of *Arabidopsis thaliana* Columbia (Col) ecotype wild-type (Col-0) and the

ascorbate deficient mutant (*vtc2*) were transformed with a redox-sensing green fluorescent protein [reduction-oxidation-sensitive green fluorescent protein] targeted either to the cytosol (c-roGFP1) or to mitochondria (m-roGFP1) using the floral dip method.^{4,12} Selected seeds expressing c-roGFP1 and m-roGFP1 were surface sterilized and plated on MS agar medium under sterile conditions and then incubated for 4 d at 4°C whereupon they were transferred to a growth chamber.

At the stage of four true leaves the seedlings were transplanted to a substrate mixture of peat/perlite/vermiculite (3:1:1; v/v/v) and maintained in a growth chamber (8 h. photoperiod; PPFD, 110 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 23.5°C). After 4 wk the experiment commenced (day 0) by imposing two watering regimes: (1) Plants were watered to saturation with Hoagland solution twice a week [substrate at approximately 90% of relative water content (RWC)] throughout the experiment [well-watered (WW) plants] and (2) water was withheld from plants [water-stressed (WS) plants] during 18 d, performing on day 18 the re-watering of these plants.

After 10 d of sampling (5-wk-old plants) two types of leaf morphologies were distinguished on plants: (M) mature; which are the first produced leaves, and which were smaller and lacking trichomes; and (Y) young developing leaves which are larger and showed a high number of trichomes per unit area compared with

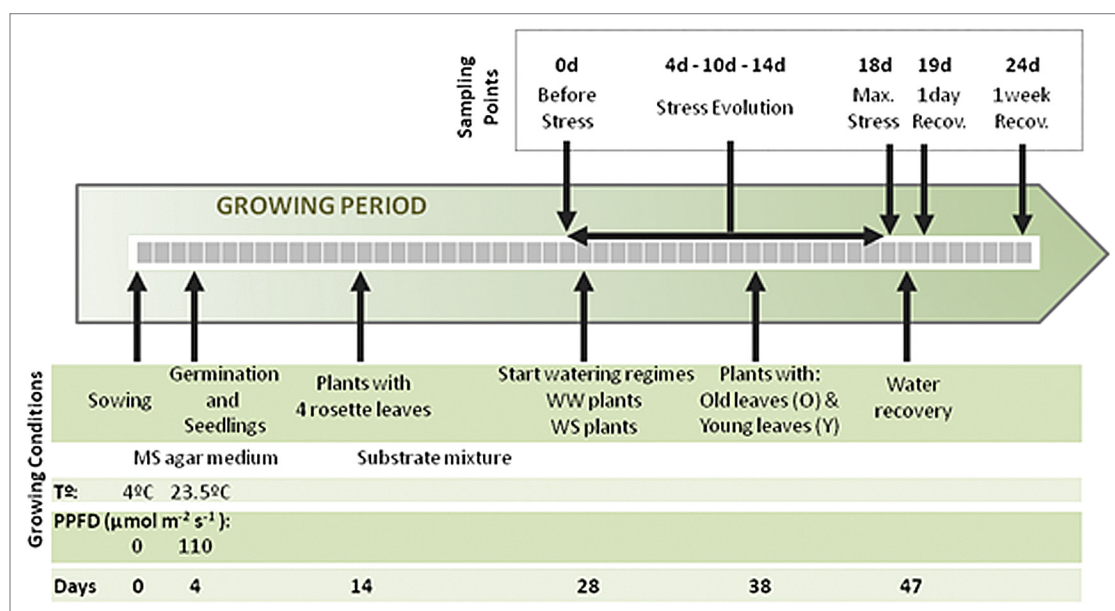


Figure 8. Diagram showing the experimental design to study the relationship between water stress and redox status in *Arabidopsis thaliana* Col-0 ecotype and *vtc2* plants expressing cytosolic or mitochondrial roGFP1.

mature leaves.³² These typologies were taken into account when making ratiometric measurements as well as in the antioxidant analysis.

After 18 d of withholding water the WS plants were re-watered to saturation and allowed to recover under the same conditions under which the WW were maintained. A diagram of the experimental design is shown in Figure 8.

Ratiometric measurements. Ratiometric measurements were made on 5-wk-mature WS and WW plants as described by,¹³ keeping track of which leaves were M or Y. Without detaching from the plant, two representative leaves of both established types were selected from each plant and directly mounted dry on a microscope slide and covered dry with a cover glass. Measurements were made according to Jiang et al.¹²

Briefly, a Nikon Diaphot Nikon FN600 microscope (Nikon) fitted with Plan Fluor 10 × /0.30 N.A. dry objectives and a Chroma Technology Phluorin filter set (exciters D410/30 and D470/20, dichroic mirror 500DCXR, emitter HQ535/50) microscope was used. Image acquisition and processing were performed using a Hamamatsu Orca-100 cooled CCD camera (Hamamatsu Corp.) and emission data (fluorescence) were collected over time using MetaFluor 6.1 image analysis software (Molecular Devices) which controlled both filters and the data collection. Fluorescence ratios were obtained by dividing the intensities obtained at 410 nm and 474 nm (i.e., 410/470). Each image was corrected for background by subtracting the intensity of an adjacent cell-free region. The 410/470 fluorescence ratios were normalized by using maximal reduced and oxidized values obtained after perfusing the leaf sequentially with adding 1 M H₂O₂ and 500 mM DTT, in order to not only drive the ro-GFP toward the fully oxidized and reduced forms, respectively, but also to prove the reversibility of the indicator. Images of these selected leaves were taken and saved within 5 min or less

and then H₂O₂ and DTT were added to attain the normalized results.

The maximal ratio under maximally oxidized conditions was set equal to 1.0 and the minimum ratio measured during maximally reduced conditions was set equal to 0.0. These normalized fluorescence ratios were then converted to redox potentials using the calibration curve generated.¹¹

Their experimentally determined data for fluorescence ratio values obtained from isolated recombinant roGFP1 titrated with DTT_{red} to DTT_{ox} solutions with calculated redox potentials were fit using a nonlinear regression equation:

$$f = y_0 + a / (1 + \exp(-(x - x_0)/b))$$

and the resulting curve ($y_0 = 0.01$, $a = 0.99$, $x_0 = -288$ and $b = 13$) was then used to calibrate these experiments.

For each measurement at least six different plants were used. From each of these plants one leaf was chosen for the redox potential measurements and the remaining leaves were used for the water relations. For processing the image data, four areas in the leaf, were selected to measure the fluorescence ratio.

Rosette water-status measurement. The aerial part of the rosettes was used for both redox measurements and to assess plant water status. $\text{RWC} (\%) = (\text{FW} - \text{DW}) / (\text{TW} - \text{DW})$, where FW is the fresh weight, TW is the turgid weight after rehydrating the samples for 24 h at 4°C in darkness and DW is the dry weight after oven-drying samples at 80°C until constant weight.

Soil water content. Gravimetric soil water content (SWC) was directly determined using the difference in soil weight immediately after the sampling (FW) and after oven drying (DW).

Analyses of reduced and oxidized forms of ascorbate and glutathione. For ascorbate and glutathione determinations, 100 mg of leaves were ground in liquid nitrogen with a mortar and pestle, followed by addition of 1 ml of extraction buffer (6% meta-Phosphoric acid; 2.5 mM EDTA) and clarified with

10 min centrifugation at 8,500 g. Re-extraction using the same conditions was performed and the two supernatants were mixed. Finally, extracts were neutralized and conveniently diluted before spectrophotometric readings, following.²¹ The reduced and oxidized forms of both ascorbate and glutathione were determined by using a 96 well quartz microplate (Hellma) and plate-reader, as described in.²⁸ The levels of ascorbate (AsA) (reduced) and dehydroascorbate (DHA) (oxidized) were determined using ascorbate oxidase (AO) and dithiothreitol (DTT), respectively, as described in.⁶ Ascorbate oxidase specifically oxidizes all reduced ascorbate in the sample. Thus the decrease in O.D. at 265 nm is related to AsA concentration. Alternatively, when the samples are incubated with DTT, DHA is reduced to AsA and the increase in O.D. is proportional to the initial DHA concentration.

Total glutathione was determined using the glutathione reductase (GR) recycling assay,³³ while for oxidized glutathione determination samples were incubated with 2-vinylpyridine.¹⁰

Statistical analyses. The data were analyzed using PASW for Windows v. 17.0.2 (SPSS Inc.). ANOVA was used to compare

mean values at a range of sampling times for RWC, glutathione and ascorbate oxidative status and content and ratiometric measurements of redox potential. The post-hoc Duncan's test was applied. Significance levels of 95% ($p < 0.05$) are indicated in figure legends. At each sampling time significantly different means are marked with different letters.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Arisi ACM, Noctor G, Foyer CH, Jouanin L. Modification of thiol contents in poplars (*Populus tremula* x *P. alba*) overexpressing enzymes involved in glutathione synthesis. *Planta* 1997; 203:362-72; PMID:9431683; <http://dx.doi.org/10.1007/s004250050202>
2. Bartoli CG, Pastori GM, Foyer CH. Ascorbate biosynthesis in mitochondria is linked to the electron transport chain between complexes III and IV. *Plant Physiol* 2000; 123:335-44; PMID:10806250; <http://dx.doi.org/10.1104/pp.123.1.335>
3. Björkman O, Badger MR, Armond PA. Response and adaptation of photosynthesis to high temperatures. In: Turner NC, Kramer PJ, eds. *Adaptation of plants to water and high temperature stress*. New York: Wiley, 1980; 233-49.
4. Clough SJ, Bent AF. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* 1998; 16:735-43; PMID:10069079; <http://dx.doi.org/10.1046/j.1365-3113.1998.00343.x>
5. Colville L, Smirnoff N. Antioxidant status, peroxidase activity, and mutants. *J Exp Bot* 2008; 59:3857-68; PMID:18849295; <http://dx.doi.org/10.1093/jxb/ern229>
6. Foyer C, Rowell J, Walker D. Measurement of the ascorbate content of spinach leaf protoplasts during illumination. *Planta* 1983; 157:239-44; <http://dx.doi.org/10.1007/BF00405188>
7. Foyer CH, Kiddle G, Verrier P. Transcriptional profiling approaches to understanding how ascorbate regulates growth and the cell cycle. In: Baginsky S, Fermie AR, eds. *Plant system biology*. Basel: Birkenhauser Publishing, 2006.
8. Foyer CH, Noctor G. Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. *Antioxid Redox Signal* 2009; 11:861-905; PMID:19239350; <http://dx.doi.org/10.1089/ars.2008.2177>
9. Foyer CH, Noctor G. Ascorbate and glutathione: the heart of the redox hub. *Plant Physiol* 2011; 155:2-18; PMID:21205630; <http://dx.doi.org/10.1104/pp.110.167569>
10. Griffith OW. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal Biochem* 1980; 106:207-12; PMID:7416462; [http://dx.doi.org/10.1016/0003-2697\(80\)90139-6](http://dx.doi.org/10.1016/0003-2697(80)90139-6)
11. Hanson GT, Aggeler R, Oglesbee D, Cannon M, Capaldi RA, Tsien RY, et al. Investigating mitochondrial redox potential with redox-sensitive green fluorescent protein. *J Biol Chem* 2003; 279:13044-53
12. Jiang K, Schwarzer C, Lally E, Zhang S, Ruzin S, Machen T, et al. Expression and characterization of a redox-sensing green fluorescent protein (reduction-oxidation-sensitive green fluorescent protein) in *Arabidopsis*. *Plant Physiol* 2006; 141:397-403; PMID:16760494; <http://dx.doi.org/10.1104/pp.106.078246>
13. Jubany-Mari T, Alegre-Battle L, Jiang K, Feldman LJ. Use of a redox-sensing GFP (c-roGFP1) for real-time monitoring of cytosol redox status in *Arabidopsis thaliana* water-stressed plants. *FEBS Lett* 2010; 584:889-97; PMID:20079738; <http://dx.doi.org/10.1016/j.febslet.2010.01.014>
14. Jubany-Mari T, Munné-Bosch S, Alegre L. Redox regulation of water stress responses in field-grown plants. Role of hydrogen peroxide and ascorbate. *Plant Physiol Biochem* 2010; 48:351-8; PMID:20199867; <http://dx.doi.org/10.1016/j.plaphy.2010.01.021>
15. Lim PO, Kim HJ, Nam HG. Leaf senescence. *Annu Rev Plant Biol* 2007; 58:115-36; PMID:17177638; <http://dx.doi.org/10.1146/annurev.arplant.57.032905.105316>
16. Mailloux RJ, Adjiteye CNK, Xuan JY, Harper ME. Crucial yet divergent roles of mitochondrial redox state in skeletal muscle vs. brown adipose tissue energetics. *FASEB J* 2012; 26:363-75; PMID:21940996; <http://dx.doi.org/10.1096/fj.11-189639>
17. Meyer Y, Belin C, Delorme-Hinoux V, Reicheld JP, Riondet C. Thioredoxin and glutaredoxin systems in plants: Molecular mechanisms, crosstalks, and functional significance. *Antioxid Redox Signal* 2012; 17:1124-60; PMID:22369066; <http://dx.doi.org/10.1089/ars.2011.4327>
18. Millar AH, Mittova V, Kiddle G, Heazlewood JL, Bartoli CG, Theodoulou FL, et al. Control of ascorbate synthesis by respiration and its implications for stress responses. *Plant Physiol* 2003; 133:443-7; PMID:14555771; <http://dx.doi.org/10.1104/pp.103.028399>
19. Mittler R, Vanderauwera S, Gollery M, Van Breusegem F. Reactive oxygen gene network of plants. *Trends Plant Sci* 2004; 9:490-8; PMID:15465684; <http://dx.doi.org/10.1016/j.tplants.2004.08.009>
20. Munné-Bosch S, Alegre L. Die and let live: leaf senescence contributes to plant survival under drought stress. *Funct Plant Biol* 2004; 31:203-16; <http://dx.doi.org/10.1071/FP03236>
21. Noctor G, Foyer CH. Simultaneous measurement of foliar glutathione, γ -glutamylcysteine, and amino acids by high-performance liquid chromatography: comparison with two other assay methods for glutathione. *Anal Biochem* 1998; 264:98-110; PMID:9784193; <http://dx.doi.org/10.1006/abio.1998.2794>
22. Noctor G. Metabolic signalling in defence and stress: the central roles of soluble redox couples. *Plant Cell Environ* 2006; 29:409-25; PMID:17080595; <http://dx.doi.org/10.1111/j.1365-3040.2005.01476.x>
23. Olmos E, Kiddle G, Pellny TK, Kumar S, Foyer CH. Modulation of plant morphology, root architecture, and cell structure by low vitamin C in *Arabidopsis thaliana*. *J Exp Bot* 2006; 57:1645-55; PMID:16720601; <http://dx.doi.org/10.1093/jxb/erl010>
24. Page MM, Robb EL, Salway KD, Stuart JA. Mitochondrial redox metabolism: aging, longevity and dietary effects. *Mech Ageing Dev* 2010; 131:242-52; PMID:20219522; <http://dx.doi.org/10.1016/j.mad.2010.02.005>
25. Pavet V, Olmos E, Kiddle G, Mowla S, Kumar S, Antoniow J, et al. Ascorbic acid deficiency activates cell death and disease resistance responses in *Arabidopsis*. *Plant Physiol* 2005; 139:1291-303; PMID:16244149; <http://dx.doi.org/10.1104/pp.105.067686>
26. Pignocchi C, Kiddle G, Hernández I, Foster SJ, Asensi A, Taybi T, et al. Ascorbate-oxidase-dependent changes in the redox state of the apoplast modulate gene transcription leading to modified hormone signaling and defense in tobacco. *Plant Physiol* 2006; 141:423-35; PMID:16603663; <http://dx.doi.org/10.1104/pp.106.078469>
27. Porters G, Horemans N, Jansen MA. The cellular redox state in plant stress biology—a charging concept. *Plant Physiol Biochem* 2010; 48:292-300; PMID:20137959; <http://dx.doi.org/10.1016/j.plaphy.2009.12.007>
28. Queval G, Noctor G. A plate reader method for the measurement of NAD, NADP, glutathione, and ascorbate in tissue extracts: Application to redox profiling during *Arabidopsis* rosette development. *Anal Biochem* 2007; 363:58-69; PMID:17288982; <http://dx.doi.org/10.1016/j.ab.2007.01.005>
29. Schwarzländer M, Fricker MD, Sweetlove LJ. Monitoring the in vivo redox state of plant mitochondria: effect of respiratory inhibitors, abiotic stress and assessment of recovery from oxidative challenge. *Biochim Biophys Acta* 2009; 1787:468-75; PMID:19366606; <http://dx.doi.org/10.1016/j.bba-bio.2009.01.020>

30. Siendones E, Gonzalez-Reyes JA, Santos-Ocana C, Navas P, Cordoba F, Cordoba F. Biosynthesis of ascorbic acid in kidney bean. L-galactono-gamma-lactone dehydrogenase is an intrinsic protein located at the mitochondrial inner membrane. *Plant Physiol* 1999; 120:907-12; PMID:10398727; <http://dx.doi.org/10.1104/pp.120.3.907>
31. Suzuki N, Koussevitzky, Mittler R, Miller G. ROS and redox signaling in the response of plants to abiotic stress. *Plant Cell Environ* 2012; 35:259-70; PMID:21486305; <http://dx.doi.org/10.1111/j.1365-3040.2011.02336.x>
32. Telfer A, Bollman KM, Poethig RS. Phase change and the regulation of trichome distribution in *Arabidopsis thaliana*. *Development* 1997; 124:645-54; PMID:9043079
33. Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal Biochem* 1969; 27:502-22; PMID:4388022; [http://dx.doi.org/10.1016/0003-2697\(69\)90064-5](http://dx.doi.org/10.1016/0003-2697(69)90064-5)
34. Veljovic-Jovanovic SD, Pignocchi C, Noctor G, Foyer CH. Low ascorbic acid in the vtc-1 mutant of *Arabidopsis* is associated with decreased growth and intracellular redistribution of the antioxidant system. *Plant Physiol* 2001; 127:426-35; PMID:11598218; <http://dx.doi.org/10.1104/pp.010141>
35. Zechmann B, Mauch F, Sticher L, Müller M. Subcellular immunocytochemical analysis detects the highest concentrations of glutathione in mitochondria and not in plastids. *J Exp Bot* 2008; 59:4017-27; PMID:18977750; <http://dx.doi.org/10.1093/jxb/ern243>