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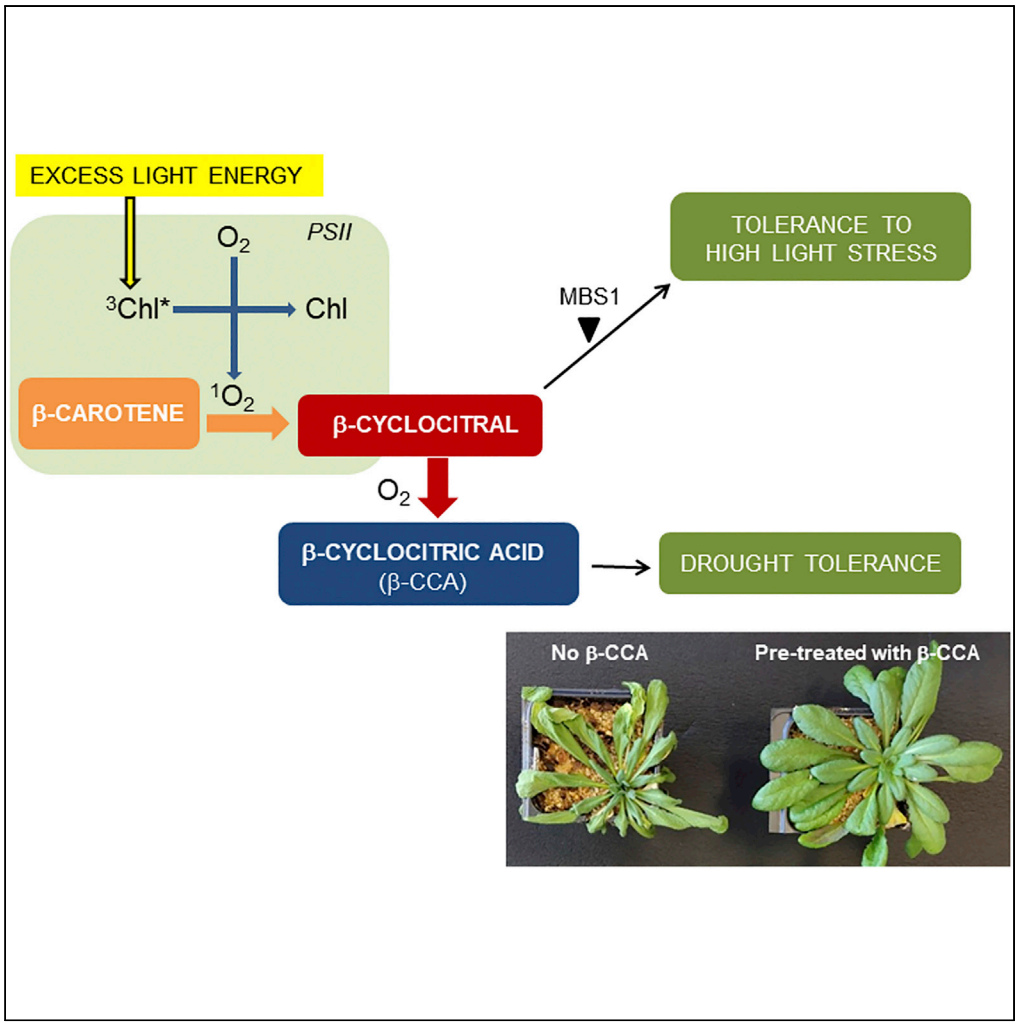

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HIGHLIGHTS

β-Cyclocitral produced by $^1O_2$ oxidation of β-carotene oxidizes to β-cyclocitric acid

β-Cyclocitric acid triggers a signaling cascade leading to drought tolerance

β-Cyclocitric acid is water soluble and offers perspectives in crop protection

The protective action of β-cyclocitric acid does not rely on stomatal closure

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SUMMARY

β-Cyclocitral (β-CC) is a volatile compound deriving from $^1O_2$ oxidation of β-carotene in plant leaves. β-CC elicits a retrograde signal, modulating $^1O_2$-responsive genes and enhancing tolerance to photooxidative stress. Here, we show that β-CC is converted into water-soluble β-cyclocitric acid (β-CCA) in leaves. This metabolite is a signal that enhances plant tolerance to drought by a mechanism different from known responses such as stomatal closure, osmotic potential adjustment, and jasmonate signaling. This action of β-CCA is a conserved mechanism, being observed in various plant species, and it does not fully overlap with the β-CC-dependent signaling, indicating that β-CCA induces only a branch of β-CC signaling. Overexpressing SCARECROW-LIKE14 (SCL14, a regulator of xenobiotic detoxification) increased drought tolerance and potentiated the protective effect of β-CCA, showing the involvement of the SCL14-dependent detoxification in the phenomenon. β-CCA is a bioactive apocarotenoid that could potentially be used to protect crop plants against drought.

INTRODUCTION

Oxidation of the carotenoid β-carotene by reactive oxygen species (ROS), especially singlet oxygen ($^1O_2$), produces various derivatives (apocarotenoids) including β-cyclocitral (β-CC) (D'Alessandro and Havaux, 2019; Ramel et al., 2012a, 2012b; Shumbe et al., 2014). This phenomenon was shown to take place in plant leaves and to be enhanced under stress conditions (Ramel et al., 2012a, 2012b). In fact, when plants are exposed to environmental constraints (i.e., drought, cold, or pathogens), which inhibit the photosynthetic activity, light energy can be absorbed in excess to what can be used by the photosynthetic processes, hence favoring transfer of electrons or excitation to oxygen and leading to ROS formation (Asada, 2006; Li et al., 2009). Singlet oxygen is produced from triplet-excited chlorophylls, mainly in the photosystem (PS) II reaction centers (Pinnola and Bassi, 2018; Krieger-Liszkay et al., 2008, Telfer, 2014; Pospíšil and Prasad, 2014). In fact, the PSII centers bind several β-carotene molecules that can scavenge $^1O_2$ molecules generated therein (Pinnola and Bassi, 2018; Ferreira et al., 2004). $^1O_2$ quenching by carotenoids proceeds by a physical mechanism that leads to thermal energy dissipation (Ouchi et al., 2010) and through a chemical quenching mechanism involving direct oxidation of the carotenoid molecule by $^1O_2$ (Stratton et al., 1993; Ramel et al., 2012b; Pinnola and Bassi, 2018). Thus, as a major site of $^1O_2$ production, PSII is also a major generator of oxidized β-carotene metabolites such as β-CC (D'Alessandro and Havaux, 2019).

β-CC (Figure S1A) is a volatile compound that was shown to act as a signal molecule in *Arabidopsis* (*Arabidopsis thaliana*), triggering changes in the expression of $^1O_2$-responsive genes and leading to acclimation to $^1O_2$ and photooxidative stress (Ramel et al., 2012a). Produced by excessive light excitation at the level of PSII in the chloroplast, β-CC can be considered as an upstream mediator in the $^1O_2$ retrograde pathway leading to acclimation. Actually, β-CC is one among several signaling metabolites that have been recently associated with chloroplast-to-nucleus retrograde signaling (Chi et al., 2015; Chan et al., 2016). However, for most of them including β-CC, the primary targets are still unknown. Moreover, as discussed elsewhere (D'Alessandro and Havaux, 2019), the β-CC-dependent pathway is distinct from other retrograde signaling pathways such as the tetrapyrrole pathway (Strand et al., 2003; Woodson et al., 2011) or the EXECUTER-mediated pathway (Lee et al., 2007). However, as the transcriptome of β-CC-treated *Arabidopsis* plants suggests an effect of the apocarotenoid on enzymes related to PAP (3′-phosphoadenosine 5′-phosphate) metabolism (D'Alessandro and Havaux, 2019), an interaction between the pathways mediated by β-CC and PAP (see Estavillo et al., 2011) is a possibility that remains to be investigated. Here, we show that β-CC is converted to β-cyclocitric acid (β-CCA) not only in water as previously reported (Tomita et al., 2016) but also *in vivo*, thus constituting one of the first steps in the β-CC-dependent signaling. Exposing plants to exogenous β-CCA induces the expression of β-CC- and $^1O_2$-responsive genes and enhances plant resistance to stress conditions such as drought. As β-CCA is a water-soluble

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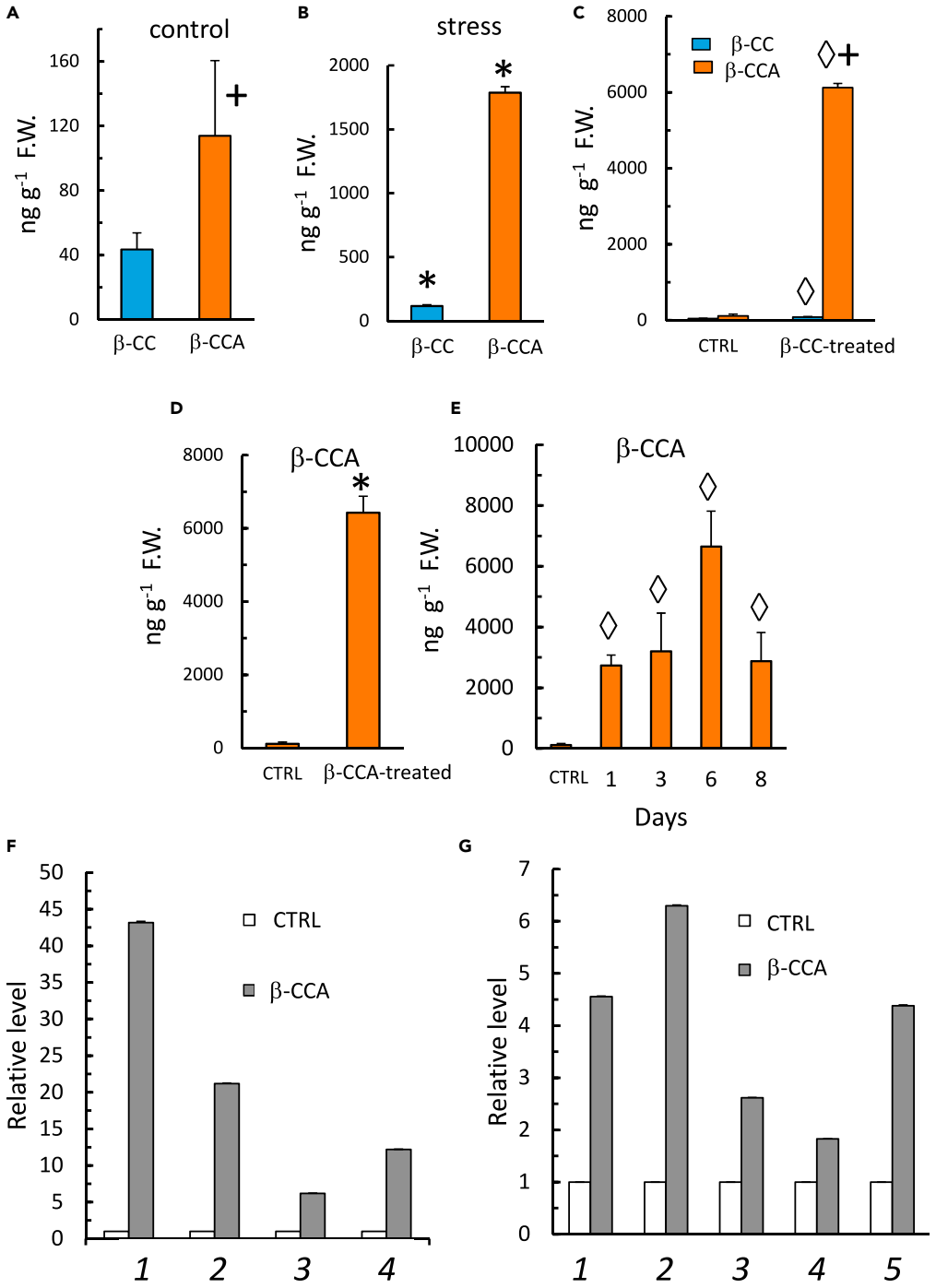

Figure 1. β-CCA Levels in Leaves of *Arabidopsis* Plants and Their Effect on Gene Expression

(A) Control, untreated plants. Data are the mean of 5 plants; error bars represent SD of the mean.

(B) Plants exposed to water stress (no watering for 7 days).

(C) Plants treated for 4 h with 100 μL volatile β-CC in a hermetically closed box. The controls were treated similarly with 100 μL water.

(D) β-CCA levels in *Arabidopsis* leaves sprayed with 50 μL/leaf of 1 mM β-CCA or of water (control). Leaves were taken 24 h after the treatment.

(E) β-CCA levels in leaves of *Arabidopsis* plants watered at time 0 with 25 mL 1 mM β-CCA solution or with watered acidified with 1 mM citric acid. Leaves were washed carefully with distilled water before the β-CCA quantification to

Figure 1. *Continued*
remove the β-CCA deposits on the leaf surfaces and hence to measure the internal concentration. Data are mean of 6 plants + SD.
(F) Expression levels of $^1O_2$- and β-CC-responsive genes analyzed by qRT-PCR in control leaves and in leaves sprayed with β-CCA. Data points are mean of 3 plants + SD.
(G) Expression levels of $^1O_2$- and β-CC-responsive genes analyzed by qRT-PCR in control plants and in plants watered with β-CCA or with 1 mM citric acid for 24 h. Data are mean values of 3 plants + SD.
In (F and G), *1* = AT3G04000, *2* = AT5G61820, *3* = AT5G63790, *4* = AT5G16970. In (G), *5* = AT3G28580. * and ◇, different from CTRL at $p < 0.01$ and $p < 0.05$ (Student's t test); +, different from β-CC at $p < 0.01$ (Student's t test).

molecule that can be easily applied to plants, e.g., through irrigation water, these results support the possibility of using this compound to boost drought resistance of crops.

RESULTS AND DISCUSSION

β-CCA Accumulates in *Arabidopsis* Leaves

β-CC can oxidize into β-CCA (2,2,6-trimethylcyclohexene-1-carboxylic acid), also known as β-cyclogeranic acid (Figures S1A and S1B). This conversion occurs spontaneously, e.g., upon addition of β-CC in water (Tomita et al., 2016), as confirmed in Figure S1C (Supplemental Information). When injected in water, β-CC disappeared within 24 h, with the concomitant appearance of β-CCA as major oxidation product (Figure S1C) (Tomita et al., 2016). The question arises as to whether oxidation of β-CC into β-CCA takes place *in vivo* too. Using gas chromatography-mass spectrometry, we were able to measure β-CCA in non-stressed *Arabidopsis* leaves, and the measured concentrations were even higher than β-CC levels (Figure 1A). This relative accumulation of β-CCA compared with β-CC was amplified under stress conditions: when plants were exposed to drought stress, the β-CC concentration rose by a factor of 3, revealing a condition of excessive light and photooxidative stress (Ramel et al., 2012a; Shumbe et al., 2017), whereas a 15-fold increase in β-CCA was observed (Figure 1B). Moreover, when plants were treated for 4 h with volatile β-CC in a closed Plexiglas box (as previously described, Ramel et al., 2012a), the increased levels of β-CC in the leaves (about 3 times) were found to be associated with a strong accumulation of β-CCA (Figure 1C), showing that the conversion of β-CC into β-CCA does take place *in vivo*. The oxidation of β-CC in water without adding any oxidizing reagent (besides dissolved O_2) was suggested by Tomita et al. (2016) to proceed according to the Baeyer-Villiger oxidation mechanism, which produces esters from ketones and carboxylic acids from aldehydes (Renz and Meunier, 1999). This β-CC-to-β-CCA conversion in *Arabidopsis* appeared to occur with a very high efficiency because the accumulation levels of β-CCA in β-CC-treated plants were much higher than the β-CC accumulation levels (Figure 1C). Therefore, we cannot exclude that β-CCA formation is facilitated by an enzyme-catalyzed reaction *in planta*, e.g., by a Baeyer-Villiger monooxygenase (van Berkel et al., 2006), as previously reported for the oxidation of castasterone to brassinolide in brassinosteroid biosynthesis (Kim et al., 2005).

When attached leaves were directly sprayed with β-CCA (Figure 1D), a strong accumulation of β-CCA was measured inside leaf tissues, indicating that this compound can readily enter the leaves. As β-CCA is soluble and stable in water contrary to β-CC, it can be easily applied to whole plants through irrigation. In Figure 1E, *Arabidopsis* plants, treated with β-CCA through the soil, showed β-CCA accumulation in the leaves. This indicates that exogenously applied β-CCA is taken up by the roots and transported to the leaves through the xylem. Whether applied through the roots or directly on the leaves, β-CCA accumulated in leaves without any significant change in the β-CC content (Figure S2). As β-CCA is directly formed from β-CC, *de novo* synthesis of β-CCA would imply an increased synthesis of β-CC, which was not observed, making improbable that the β-CCA treatment triggered β-CCA synthesis rather than β-CCA fluxes from the soil to the plant tissues.

β-CCA Induces Changes in Gene Expression and Increases Plant Tolerance to Drought

The expression of a number of genes that were previously identified as responsive to β-CC (Ramel et al., 2012a) was analyzed by qRT-PCR before and after application of β-CCA by spraying attached leaves or by watering plants, including AT3G04000 (*ChlADR*), AT5G61820 (unknown), AT5G63790 (*ANAC102*), AT5G16970 (*ALKENAL REDUCTASE AER*), and AT3G28580 (*AAA + ATPASE*). Both direct application of β-CCA on leaves and watering of plants with a solution of β-CCA induced a strong upregulation of the selected genes (Figures 1F and 1G), indicating that β-CCA acts as a signaling molecule triggering transcriptomic changes. The gene upregulation levels were lower in plants watered with β-CCA relative to leaves directly sprayed with β-CCA, as were the accumulation levels of β-CCA inside the leaf tissues (24 h after treatment).

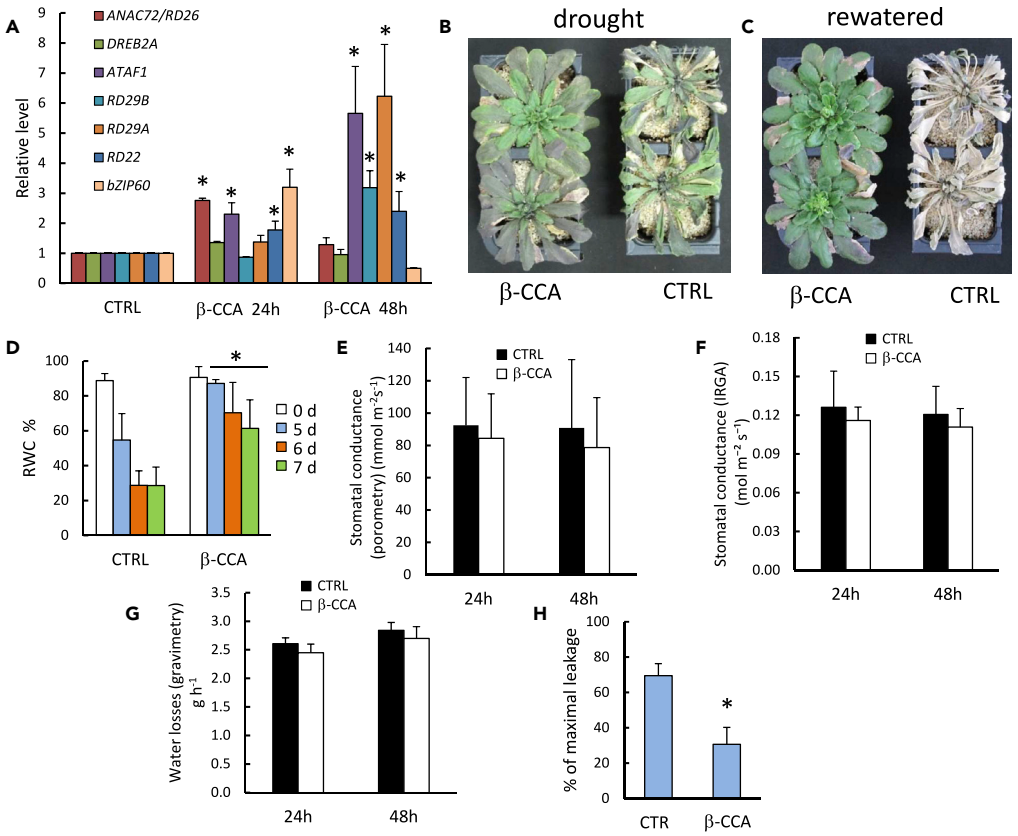

Figure 2. β-CCA-Induced Protection of *Arabidopsis* Plants against Drought Stress

(A) Expression levels of water stress-responsive genes in leaves of *Arabidopsis* plants watered with β-CCA or with citric acid (data are means of 3 plants +SD).

(B) Picture of *Arabidopsis* plants pre-treated with β-CCA or with citric acid (CTRL) and then subjected for 7 days to drought stress imposed by withdrawing watering.

(C) Picture of the same plants 10 days after re-watering with plain water.

(D) RWC of plants subjected to water stress after pre-treatment with β-CCA or with acidified water (data are mean values of 6 leaves + SD).

(E and F) Stomatal conductance measured by porometry (data are mean values of 10 leaves + SD) (E) or by infra-red gas analysis (IRGA) (data are mean values of 5 plants + SD) (F).

(G) Losses of water by *Arabidopsis* plants (treated or untreated with β-CCA) during water stress (data are mean of 3 plants + SD).

(H) Increased membrane permeability in *Arabidopsis* leaves, as measured by electrolyte leakage, after 7 days of water stress (data are mean of 3 plants + SD). *, Different from CTRL at $p < 0.01$ (Student's t test).

A previous microarray-based transcriptomic study revealed that a number of water stress-responsive genes are also inducible by β-CC (Ramel et al., 2012a). The same phenomenon was observed for β-CCA by qRT-PCR analyses (Figure 2A): the drought marker genes *ATAF1* (ANAC002, AT1G01720), *RD29A* (AT5G52310), *RD29B* (AT5G51180), *RD22* (AT5G25610), and *bZip60* (AT1G42990) (Yamaguchi-Shinozaki and Shinozaki, 1993; Lu et al., 2007; Xiong et al., 1999; Wang et al., 2017) were induced by β-CCA in the absence of any water stress. However, not all drought marker genes responded to β-CCA because *DREB2A* (AT5G05410), a transcription factor functioning in water stress response (Sakuma et al., 2006), was not induced. *RD26* (*ANAC072*, AT4G27410) and *bZIP60* (AT1G42990) showed a different response to β-CCA compared with the other genes, as their induction was transient. Nevertheless, our results indicate that the response elicited by β-CCA overlaps, at least partially, with the genetic response to water stress. This response may reflect the role of $^1O_2$ in water stress (Koh et al., 2016). This prompted us to analyze the effect of β-CCA on *Arabidopsis* plants exposed to drought stress. Following irrigation of *Arabidopsis* plants with water containing or not containing β-CCA (same pH), water stress was induced by withdrawing irrigation. After 7 days, control plants showed clear symptoms of stress and dehydration (Figure 2B). These

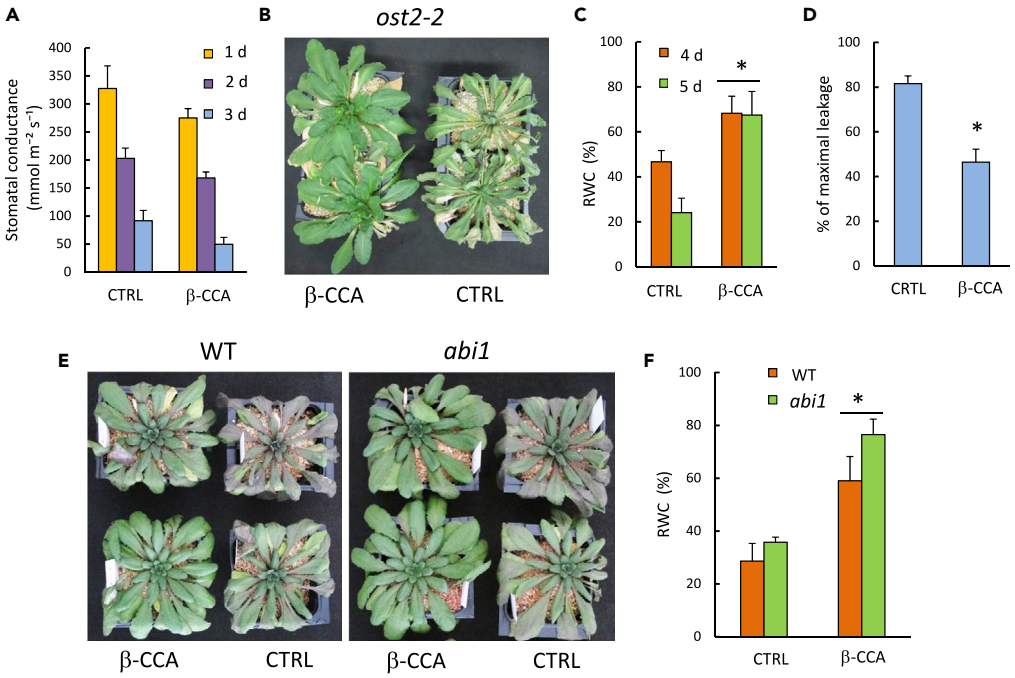

Figure 3. β-CCA-Induced Protection of *Arabidopsis ost2-2* and *abi1* Mutant Plants against Drought Stress

(A) Stomatal conductance (data are mean values of 10 leaves + SD) of WT or *ost2-2* plants pre-treated with β-CCA or with water and then subjected to water stress.

(B) Picture of *ost2-2* mutant plants pre-treated with β-CCA or with water (CTRL) and then subjected to 5 days drought stress.

(C) RWC (data are mean of 6 leaves + SD).

(D) Membrane permeability in *ost2-2* mutant leaves measured by ion leakage after 5 days of water stress (data are mean values of 6 leaves).

(E) Picture of *abi1* mutant plants pre-treated with β-CCA or with water (CTRL) and then subjected to 5 days drought stress.

(F) RWC of leaves (mean values of 6 leaves). *, Different from CTRL at $p < 0.01$ (Student's t test).

symptoms were strongly attenuated in plants pretreated with β-CCA. After rewatering with plain water, control plants did not recover and died, whereas β-CCA-treated plants recovered and were fully turgescent (Figure 2C). The leaf relative water content (RWC) was much higher in plants treated with β-CCA throughout the water stress treatment compared with control plants (Figure 2D). The protection of *Arabidopsis* by β-CCA against drought stress was confirmed in other plant species such as pepper (*Capsicum*), pansy flower plants (*Viola tricolor*) (Figure S3), and tomato (*Solanum lycopersicum*, see below).

Furthermore, we checked the possible effect of β-CCA on soil water retention. The pots were watered with water containing 0 or 1.5 mM β-CCA, and the soil was then let to dehydrate for several days. No significant difference was found in the rate of soil drying between the two conditions (Figure S4), indicating that the β-CCA-induced increase in plant drought tolerance was not due to an effect on the soil itself.

β-CCA Does Not Induce Stomatal Closure

Typical responses of plants to water stress are stomatal closure mediated by the apocarotenoid abscisic acid (ABA), hence reducing transpiration and water losses (Chaves et al., 2003; Munemasa et al., 2015; Nguyen et al., 2016), osmotic adjustment (Umezawa et al., 2006; Blum, 2017), and jasmonate signaling (Kim et al., 2017). Although β-CCA is an apocarotenoid as well as ABA, the protective role of beta carotene against drought stress does not rely on stomatal regulation. In fact, stomatal closure was not induced by β-CCA (Figures 2E and 2F), and transpiration was similar in β-CCA-treated and β-CCA-untreated plants (Figure 2G). We also examined the effects of β-CCA on the *ost2-2 Arabidopsis* mutant that is impaired in stomatal regulation due to a constitutive activation of a plasma membrane ATPase (Merlot et al., 2007) and on the *abi1* mutant affected in ABA signaling (Leung et al., 1997). As expected, stomatal

conductance of *ost2-2* leaves was enhanced compared with wild-type (WT) leaves (Figures 3C versus 2E), and water stress developed much more rapidly in *ost2-2* plants relative to WT plants when water irrigation was stopped (Figures 3B versus 2D). However, the protective action of β-CCA was confirmed in the mutant: leaf dehydration and loss of turgescence were less pronounced in β-CCA-treated *ost2-2* plants (Figures 3A and 3B). As with WT plants (Figure 2), the RWC of *ost2-2* mutant plants was preserved by β-CCA during water stress (Figure 3B). When in the col-0 background, the *abi1* mutant is not more sensitive to drought treatments than WT (Harb et al., 2010), but the tolerance of *abi1* to drought was noticeably increased by β-CCA (Figures 3E and 3F). The protection of *ost2-2* and *abi1* by β-CCA confirms that the mode of action of β-CCA does not rely on ABA signaling and the associated stomatal closure. We also measured stomatal density as well as the stomatal index before and after β-CCA treatment, because a moderate reduction of this factor can improve water use efficiency (Dunn et al., 2019). No significant difference was observed between β-CCA-treated and β-CCA-untreated plants for these parameters (Figure S5).

Also, plant response to water stress usually involves synthesis of osmoprotectants, such as ammonium compounds, sugar, sugar alcohols, and amino acids (Umezawa et al., 2006; Blum, 2017), which permit the maintenance of turgor pressure under water stress conditions, thus preserving vital functions. We checked whether accumulation of β-CCA in leaves could bring about substantial changes in leaf osmotic potential Ψ_π. However, the data of Figure 4A show that the protective function of β-CCA against drought stress does not rely on this phenomenon. Indeed, Ψ_π (−0.815 MPa) of leaves taken from plants watered with β-CCA did not differ significantly from Ψ_π of control leaves (−0.786 MPa). Finally, the involvement of a jasmonic acid-dependent mechanism (Kim et al., 2017) in the β-CCA effect was excluded. In fact, the *Arabidopsis* mutant *coi1*, which lacks the jasmonate receptor COI1, although more sensitive than the WT, responded to β-CCA like the WT, by an increase in drought tolerance (Figures 4B and 4C).

Interestingly, a recent work by Dickinson et al., 2019 has shown that β-CC could act as a root growth regulator. A stimulating effect on root growth can potentially increase drought tolerance by enhancing water uptake by the plant (Wasaya et al., 2018). To check this possibility, we grew *Arabidopsis* seedlings in Petri dishes on solid medium containing 0, 15, and 150 nM β-CCA. Root growth was measured on 15-day-old seedlings (Figure 4D). A slight, but statistically significant, increase in root length was found to occur at 150 nM β-CCA. However, the effect of β-CCA on root growth is noticeably lower than the reported effect of β-CC at similar concentrations (Dickinson et al., 2019). Importantly, a sealing method allowing gas exchange (3M tape) was possible in this experiment thanks to the water solubility of β-CCA, and this difference may explain the lower efficiency of β-CCA compared with that of β-CC, as commented in D'Alessandro and Havaux, 2019. Although the short-term nature of the drought stress experiments of Figures 2 and 3 (a few days) implies limited changes in root development, one cannot completely exclude that changes in root development participate in the drought tolerance of β-CCA-treated plants.

The Protective Effect of β-CCA Is Independent of *MBS1* and Is Potentiated by *SCL14* Overexpression

β-CCA protected cell membranes against damage under drought stress, as indicated by a much lower leakage of electrolytes by leaves from β-CCA-treated plants compared with untreated plants, in both the WT and *ost2-2* backgrounds (Figures 2H and Figure 3D). It is thus possible that β-CCA induces a cellular defense mechanism that reinforces membrane stability and resistance to ROS. It is likely that this protection against membrane disruption during water stress contributed to maintaining leaf water content and enhancing drought tolerance (Farooq et al., 2009; Premachandra et al., 1991; Tripathy et al., 2000). The preservation of cell membrane stability is anyway an effect recalling β-CC-induced acclimation to photooxidative stress (Ramel et al., 2012a). Unfortunately, the β-CC-dependent signaling pathway is still largely unknown. Nevertheless, the roles of *METHYLENE BLUE SENSITIVITY 1* (*MBS1*) and of the SCARECROW LIKE 14 (SCL14)-dependent detoxification pathway in response to β-CC have been recently described (Shumbe et al., 2017; D'Alessandro et al., 2018). MBS1 is a cytosolic zinc finger protein essential for regulating the expression of $^1O_2$-responsive genes (Shao et al., 2013). In the *mbs1* mutant that lacks the MBS1 protein, the expression of $^1O_2$ marker genes was shown to be markedly deregulated (Shao et al., 2013). For some genes, induction by β-CC was blocked, whereas the induction of other genes was enhanced (Shumbe et al., 2017). When exposed to drought stress, the *mbs1* mutant behaved as WT, with β-CCA providing a marked protection against water stress deprivation (Figures 5A and 5B). We can therefore conclude that the MBS1-dependent signaling pathway, triggered by β-CC and essential for the β-CC-induced acclimation to photooxidative stress, is different from the β-CCA-induced signaling pathway leading to drought

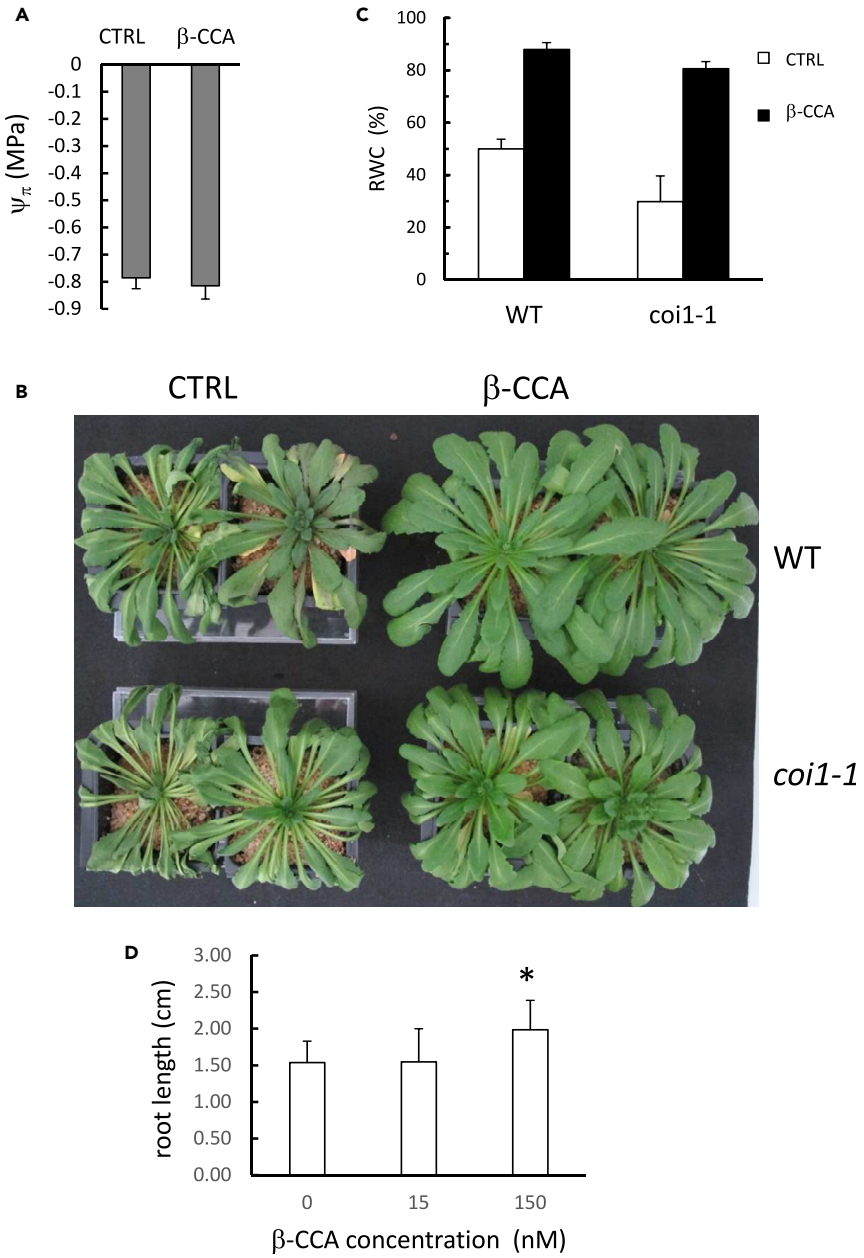

Figure 4. The β-CCA Protective Effect Does Not Require Osmotic Adjustment or Jasmonate Signaling and Is Associated with Limited Changes in Root Growth

(A) Leaf osmotic potential of *Arabidopsis* plants watered with 1 mM β-CCA for 48 h. CTRL, control (data are mean of 8 leaves + SD).

(B). Effect of water deprivation on WT *Arabidopsis* plants and on *coi1* mutant plants pre-treated with 0 or 1 mM β-CCA.

(C) RWC of the leaves (data points are mean values of 6 leaves + SD).

(D) Root length of *Arabidopsis* seedlings grown for 15 days in Petri dishes with 0, 15, and 150 nM β-CCA. Data are mean values of 16–23 seedlings + SD. *, Different from CTRL at $p < 0.01$ (Student's t test).

stress resistance. Consistently, drought-related genes whose expression was observed to be upregulated by β-CCA (Figure 2A), were also induced in the *mbs1* mutant (Figure 5C). It is therefore possible that β-CCA mediates a branch of the β-CC signaling pathway, whereas MBS1 regulates another (or several other) branch(es). Moreover, in contrast with β-CC (Ramel et al., 2012a), β-CCA did not provide any protection against photooxidative stress induced by excessive light (Figure S6), which has been previously shown

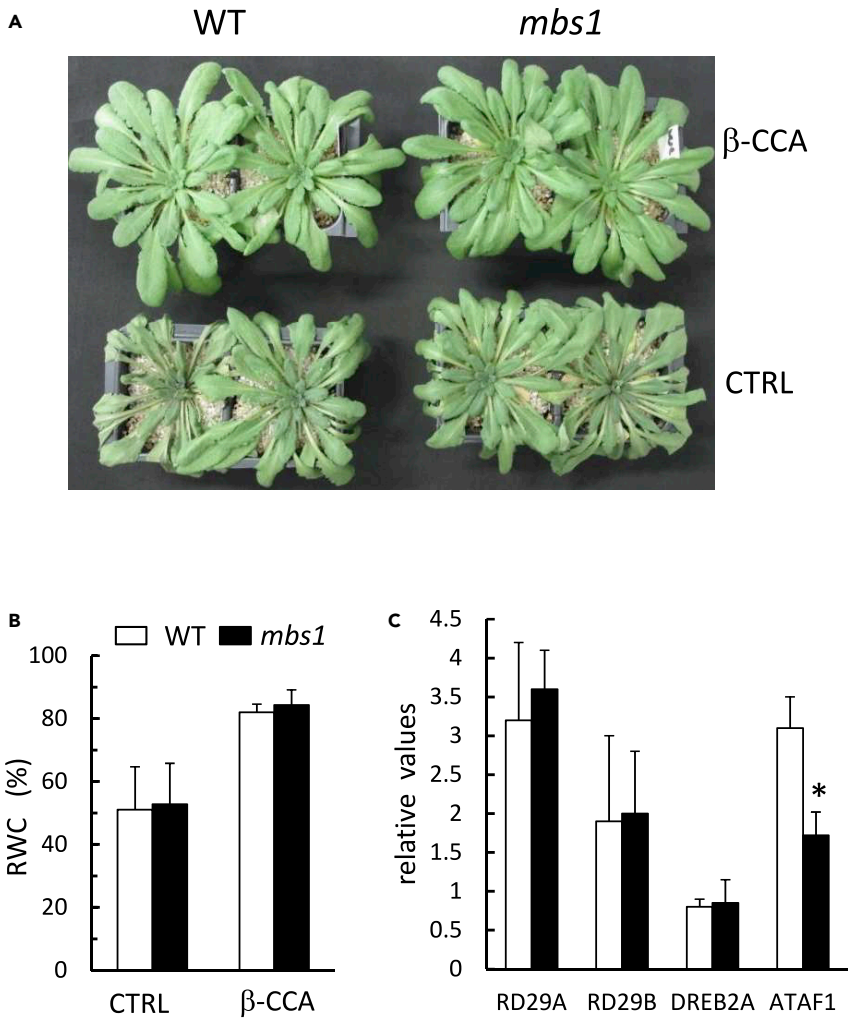

Figure 5. The *mbs1* Mutation Does Not Suppress the Protective Effect of β-CCA against Drought Stress

(A) Picture of the plants (WT and mbs1 mutant) pre-treated with β-CCA or with water (CTRL) and exposed to water stress for 7 days.

(B) RWC of WT and mbs1 leaves after 7 days of water stress (mean values of 6 leaves + SD).

(C) Expression levels of several drought-responsive genes (data points are mean of 3 plants + SD). *, Different from CTRL at $p < 0.01$ (Student's t test).

to depend on MBS1 and on the SCL14-dependent detoxification pathway (Shumbe et al., 2017; D'Alessandro et al., 2018). However, the lack of protective effect of β-CCA against high light stress could also be linked to an effect of light on the β-CCA concentration. As shown in Figure S7, the β-CCA levels in control (unstressed) leaves were substantially reduced after 24 h in high light (1,500 μmol photons m^{-2} s^{-1} at 7°C air temperature). Similarly, in β-CC-treated plants that accumulated high amounts of β-CCA, the high light treatment brought about a marked reduction of the compound. This suggests a rapid degradation or metabolization of β-CCA at elevated light intensities, which may play a role in the inefficiency of β-CCA to enhance plant tolerance to high light stress.

We tested the involvement of the more recently described SCL14-dependent branch of the β-CC response (D'Alessandro et al., 2018), by the use of the *scl14* knockout mutant and of the OE:SCL14-overexpressing line. The mutant line showed no difference with the WT in response to the β-CCA treatment (Figure S8), whereas the SCL14-overexpressing line did show an enhanced resistance to drought stress already when treated with water, and even stronger drought tolerance when treated with β-CCA (Figures 6A and 6B). In fact, the RWC of WT plants treated with β-CCA and of OE:SCL14 plants treated with water or

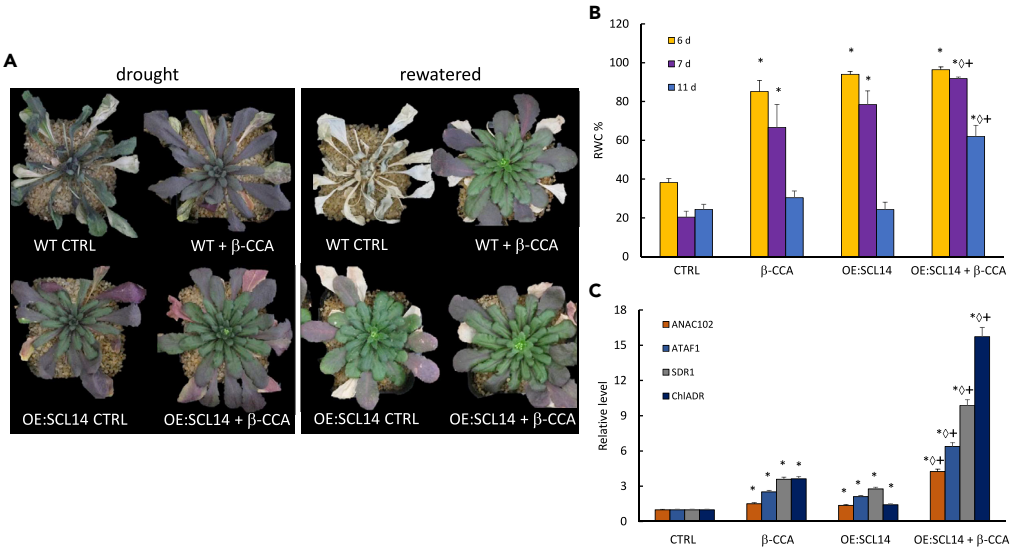

Figure 6. The β-CCA Protective Effect Was Enhanced by Overexpressing SCL14

(A) Picture of the plants (WT and OE:SCL14) pre-treated with β-CCA or with water (CTRL) and exposed to water stress for 11 days and after rewatering.

(B) RWC of WT and OE:SCL14 leaves after 6, 8, or 11 days of water stress (mean values of 6 leaves per point + SD).

(C) Expression levels of marker genes for the SCL14 response (mean values of 3 plants + SD). *, Different from CTRL at $p < O.01$; ◇, different from β-CCA treatment at $p < 0.01$; +, different from OE:SCL14 at $p < 0.01$ (Student's t test).

with β-CCA after 7 days of water withdrawal was markedly higher (70%–90% RWC) than that of WT plants treated with water (20% RWC). Furthermore, the overexpression of SCL14 and the treatment with β-CCA had an additive effect, because OE:SCL14 plants treated with β-CCA showed an RWC of 60% after 11 days of water withdrawal, indicating an almost doubled resistance compared with that of WT plants treated with water or with β-CCA. SCL14 is a GIBBERELLIC-ACID INSENSITIVE (GAI), REPRESSOR of GA1 (RGA), and SCARECROW (SCR) (GRAS) protein involved in the regulation of the xenobiotic detoxification response (Fode et al., 2008) and more recently described in the response to photooxidative stress under excessive light (D'Alessandro et al., 2018). We, thus, quantified the expression levels of four reporter genes in the SCL14-dependent response: *ANAC102*, *ATAF1*, *SDR1*, and *ChlADR*, by qRT-PCR (D'Alessandro et al., 2018; Fode et al., 2008). The four reporters were slightly induced at 24 h of treatment with β-CCA, to levels similar to the ones found in the OE:SCL14, and only the treatment of the SCL14-overexpressing lines with β-CCA generated a marked induction of the detoxification pathway (Figure 6C). The weak induction of this pathway by 24-h treatment with β-CCA is in line with the lack of protection of this treatment to excessive light conditions (Figure S6). At the same time, we cannot exclude that this pathway becomes important later in the stress, taking into account that drought stress is not yet present at 24 h. Especially considering that although the *scl14* mutant line showed fitness comparable with WT under drought stress (Figure S8), it is part of a multigenetic family: 33 GRAS proteins are encoded by the *Arabidopsis* genome (Bolle, 2004). Furthermore, at least two other members are present in the more stringent LISCL class of GRAS proteins: AtSCL9 and AtSCL11 (Xu et al., 2015), and AtSCL33 was suggested to be a paralog of SCL14 (Fode et al., 2008). On the other hand, the amazing fitness of OE:SCL14 plants both under control conditions and when treated with β-CCA highlights the role of SCL14-dependent cellular detoxification in plant protection from drought stress. Interestingly, rice plants overexpressing the OsGRAS23 protein, a homolog of AtSCL14, AtSCL11, and AtSCL9, have already been reported as resistant to drought stress (Xu et al., 2015), supporting our results in *Arabidopsis*, and further encouraging the use of β-CCA on these genotypes.

Conversely to β-CCA, pre-exposure of plants for 4 h to an atmosphere containing volatile β-CC led to a strong upregulation of the SCL14-dependent response (D'Alessandro et al., 2018). Similar to OE:SCL14 plants, the treatment with β-CC led to an enhancement of drought tolerance (Figure S9), as did β-CCA. We can, therefore, hypothesize that the strong induction of the SCL14 pathway by β-CC, together with the generation of β-CCA, could lead to an even stronger response than β-CCA by itself, although the

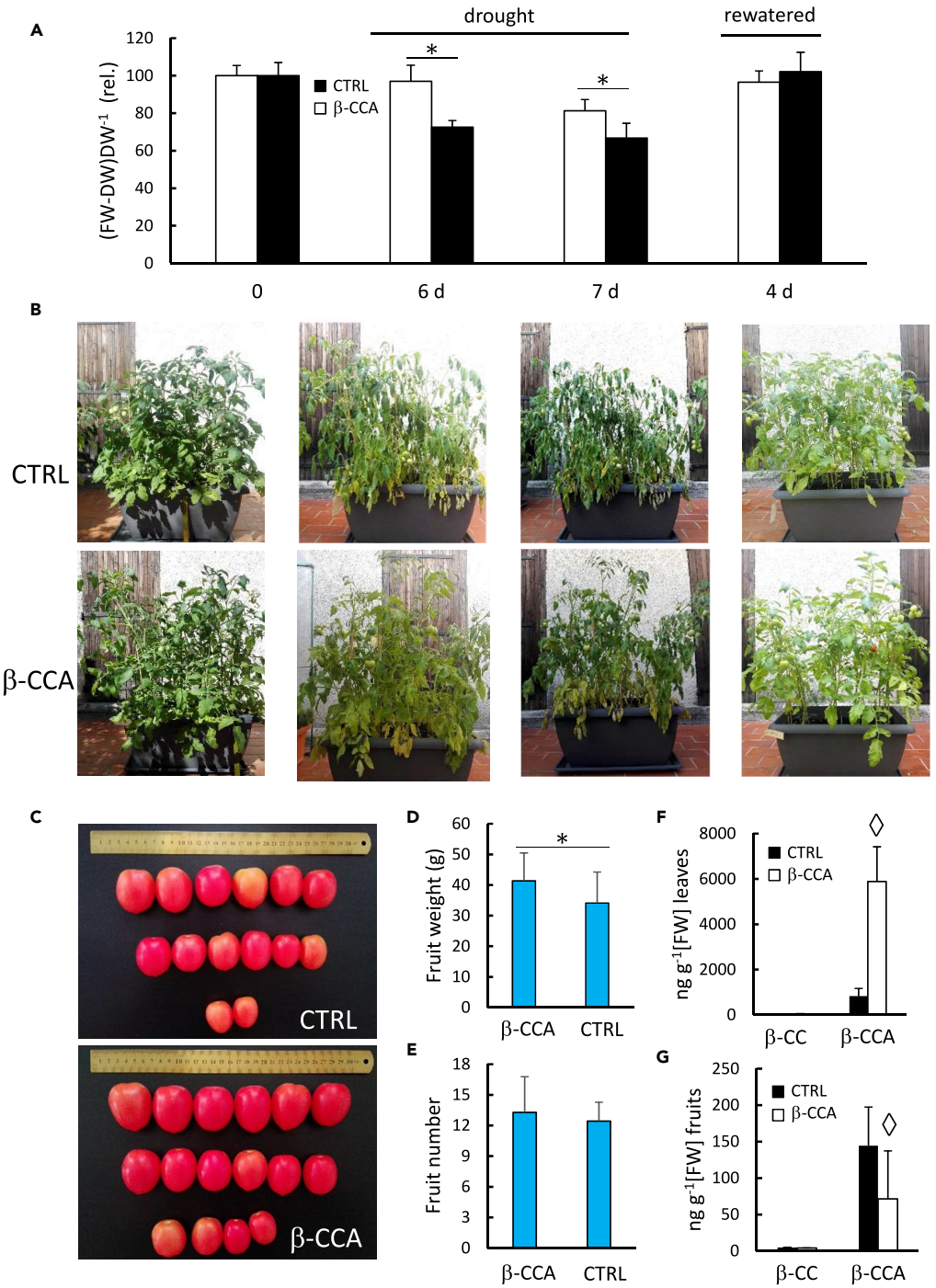

Figure 7. β-CCA Protects Tomato Plants during Water Stress in Outdoor Experiments

(A) Leaf water content of tomato plants (pre-treated with β-CCA or with water (CTRL)) during water stress (6 and 7 days after stopping watering) and recovery (4 days after re-watering) (data are mean values of 8 leaves + SD). The turgid weight was not measured in this experiment, and consequently water content (not RWC) was expressed as (FW-DW)/DW where DW is the dry weight and FW is the fresh weight. Data are expressed relative to the water content at time 0.

(B) Picture of the plants pre-treated with β-CCA or with water (CTRL) and exposed to water stress for 6 and 7 days.

(C) Tomato fruits from the first and second truss were harvested when ripe, from plants treated or untreated with β-CCA and exposed to water stress for 8 days (30 fruits).

(D) Average weight of the harvested tomato fruits (30 fruits).

Figure 7. *Continued*

(E) Average number of fruits per plant (data points are mean values of 8 plants + SD).

(F) and (G) β-CC and β-CCA levels in tomato leaves (mean values of 3 leaves + SD) (F) and in tomato fruits (mean values of 3 fruits + SD) (G). * and ◇ different at $p < 0.01$ and $p < 0.05$ (Student's t test).

volatile nature of β-CC could compromise its use in the field. These results confirm that the acclimatory response triggered by β-CCA is only part of the response induced by β-CC, whereas β-CC triggers the β-CCA response indeed.

The Anti-drought Effect of β-CCA Is a Conserved Mechanism

The protection elicited by β-CCA is conserved in several plant species such as pepper (*Capsicum*), pansy flower plants (*Viola tricolor*) (Figure S3), and tomato (*Solanum lycopersicum*). In fact, we have exposed tomato plants to a rather moderate drought stress in outdoor experiments to illustrate the potential of applications of this compound (Figure 7). Similar to *Arabidopsis*, leaves of β-CCA-treated tomato plants retained more water (Figure 7A) and showed less symptoms of leaf dehydration during drought stress (Figure 7B) than untreated plants. After recovery from the water stress period, tomato fruit size of β-CCA-treated plants was noticeably enhanced compared with that of fruits of control plants: the fruit size and weight were around 30% higher (Figures 7C, 7D, and S10), whereas the number of tomatoes was not affected (Figure 7E). The experiment of Figure 7 shows that the protective effect of β-CCA takes place also under outdoor conditions when drought is combined with temperature and light changes and can have marked beneficial effects on plant productivity. Furthermore, similarly to what we observed with *Arabidopsis*, leaves of tomato plants irrigated with β-CCA-containing water accumulated high amounts of β-CCA (Figure 7F). Interestingly, this accumulation was not found in the fruit flesh, which contained similar β-CCA levels in treated and untreated plants (Figure 7G).

Conclusions

We have identified β-CCA as a signaling molecule downstream of the apocarotenoid β-CC, which triggers the tolerance of plants toward drought stress by activating a branch of the β-CC-dependent signaling mechanism. In addition, the use of β-CCA in the SCL14- or OsGRAS23-overexpressing plants could further boost the phytoprotective effect. This work provides thus a protective agent that could be exploited to increase plant tolerance to drought using simple application procedures. As shown with tomato fruits, the use of β-CCA under natural conditions enhanced biomass productivity after a water stress period. Previous attempts to enhance plant tolerance to drought by application of exogenous compounds essentially concern the apocarotenoid phytohormone ABA (Waterland et al., 2010; Wei et al., 2015) or its synthetic agonists, quinabactin (Okamoto et al., 2013), which can reduce transpirational water losses through stomatal closure (Munemasa et al., 2015). β-CCA has a different mode of action (Figures 2 and 3) and possesses some advantages over those anti-transpirants, including low cost, stability, and protective action independent of stomatal regulation, thus limiting the effects on carbon acquisition. In relation with the latter feature, 3-week application of β-CCA was found to have no inhibitory effect on plant growth under unstressed conditions (Figure S11), indicating that this compound is not harmful to plants in the long term, at the applied concentrations. This is at variance with ABA, which was reported to reduce the growth rate of shoots both under osmotic stress and under normal conditions (Agehara and Leskovar, 2012, 2014; Meguro and Sato, 2014). Acetate is another metabolite that was recently shown to provide some protection against drought stress (Kim et al., 2017). However, its phytotoxicity is well known and is even exploited in the elaboration of herbicides (Abouziena et al., 2009), and this may limit its applicability as a phytoprotector. Independently of the molecular mechanisms that remain to be clarified, the protective effects of β-CCA reported here show that apocarotenoids different from ABA are involved in drought tolerance and are potential anti-drought agents.

Limitations of the Study

The protective action of β-CCA against the damaging effects of drought stress has been shown here in several plant species and under different environmental conditions. These findings should be completed in the future by field experiments, which should also include monocot crops such as wheat, rice, and maize. β-CCA is a signaling molecule regulating the expression of nuclear genes, finally enhancing plant tolerance to drought stress, but the exact mechanism behind this resistance remains to be clarified. In particular, the

involvement of the SCL14-dependent detoxification pathway in response to β-CCA and how a higher leaf water content is kept in β-CCA-treated plants remain to be deciphered in more detail.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.08.003.

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AUTHOR CONTRIBUTIONS

S.D. and M.H. designed research, analyzed and interpreted the experimental data, and wrote the manuscript. S.D. performed most experiments. Y.M. performed leaf osmotic potential measurements. B.L. performed the GC-MS analyses with S.D.

DECLARATION OF INTERESTS

CEA has submitted a patent on behalf of S.D. and M.H. on aspects of the findings.

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ISCI, Volume *19*

Supplemental Information

The Apocarotenoid β-Cyclocitric Acid Elicits Drought Tolerance in Plants

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Supplemental Information

Transparent Methods

Plant growth and stress treatment. Wild type (WT, ecotype Col 0), *ost2-2, mbs1, scl14* and *abi1* mutant *Arabidopsis thaliana* lines and the *SCL14*-overexpressing line (OE:SCL14) were grown for 5 weeks in short-day conditions (8h/16h, day/night) under a moderate photon flux density (PFD) of ~150 µmol photons m^{-2} s^{-1}, controlled temperature (20 °C/18 °C, day/night) under a relative air humidity of 65 %. For the drought experiments, Arabidopsis plants were watered with 25 ml per pot of water acidified with citric acid (1.5 mM) or of water containing 1.5 mM β-CCA. Water stress was subsequently applied by stopping watering. Citric acid was used to acidify water so as to expose control plants and β-CCA-treated plants to the same pH (pH ~4). We can therefore exclude that the plant responses to β-CCA are due to a pH effect. However, no difference was observed between control plants watered with acidified water and not-acidified water in terms of drought tolerance. Pepper and pansy plants were bought on the local market and acclimated in a greenhouse for 1 week. Drought stress was applied by stopping watering after the treatment with 500 ml water or 0.15 mM β-CCA per pot. Tomato plants (*Solanum lycopersicum,* cultivar Rio grande) were grown in 80-L pots (8 plants per pot). When fruits in first truss were mature and started changing their color, drought stress was applied by stopping watering after the treatment with 4 L water or 1.5 mM β-CCA per pot. Treatments of Arabidopsis plants with volatile β-CC were done in transparent airtight plexiglass box (Ramel et al., 2012).

β-cyclocitral oxidation. 1 ml of pure β-cyclocitral (Sigma-Aldrich) was transferred in 1 L of bi-distilled water, in a closed container, and agitated for 1 d. Then the container was opened and agitated for 1 additional day. 50 µl samples of the solution were taken at different times for β-CCA analyses.

GC/MS measurements. Although β-CC is a volatile compound, emission of β-CC by photosynthetic organisms is very low (Garcia-Plazaola et al. 2017) indicating that it is trapped in the plant tissues. So, solvent extraction was used to quantified β-CC and β-CCA. The lipid fraction was extracted from the samples (aqueous solutions or about 500 mg plant tissues) in 4 ml *tert*-Butyl methyl ether plus 1 ml 1mM HCl, containing 4-nonanol as internal reference

(10 μg). After centrifugation, the supernatant was collected, evaporated and analyzed by GC-MS. β-CC and β-CCA were quantified on the most probable ion (m/z 137 and 153, respectively) in SIM analyses (Ramel et al., 2012). The mass spectrum of β-CCA can be found in (Tomita et al., 2016). Pure β-CCA can be produced as reported in (Tomita et al., 2016).

Stomatal conductance measurements. Stomatal conductance was analyzed on at least two leaves per plants on six plants per condition, using an AP4 diffusive porometer (Delta-T Devices, Cambridge, UK). Two readings were taken per leaves and averaged. Measurements were made on the abaxial leaf surface between 1 h and 2 h after the start of the illumination. Stomatal conductance measurement by IRGA were performed with a LI-COR 6400 (LiCor Inc., Lincoln, NE, USA) equipped with a clamp-on leaf cuvette (6400-40 Leaf Chamber Fluorometer; LiCor Inc.). Leaf temperature was maintained at 22 °C, and leaf-to-air VPD was at 2 kPa. A 10% blue and 90% red light was provided from a LED array on top-side cuvette and set to 500 μmol m^{-2} s^{-1}. Stomatal conductance was analyzed on one leaf per plant on six plants per condition, after 30 minutes of equilibration.

Relative water content (RWC). Six leaf disks per condition were cut from leaves of β-CCA-treated or -untreated plants and weighted (FW). For measuring the turgid weight (TW), samples were submerged with bi-distilled water and left at 4°C for 16 hours. Dry weight (DW) was measured after 16h at 70°C in a ventilated oven. RWC was measured following the formula (FW-DW)/(TW-DW) x 100.

Ion Leakage. Cell membrane stability can be estimated by the use of electrolyte leakage method (Bajji et al., 2002). Six leaves per condition were cut from β-CCA-treated or -untreated plants and weighted (FW), and placed in 25 ml of bi-distilled water. Conductivity of the solution was measured with a DIST-5 Hanna conductometer after 2 h of mild agitation and after boiling the samples. The values were normalized on the FW and the values after boiling are reported as the maximal value.

Leaf osmotic potential. Leaf osmotic potential was measured on freeze-thawed leaf discs using C-52 psychrometer chambers and Psypro control unit (Wescor, Logan, UT, USA). Leaf discs of 7 mm diameter were placed in sealed plastic tubes, frozen in liquid nitrogen and kept

at -80 °C. For measurements, the discs were defrosted for 2 min and transferred onto sample holders of psychrometer chambers. To speed up the equilibration time of water potential in the chambers, leaf discs were pierced several times before enclosure (Kikuta and Richter, 1992).

RNA isolation and qRT-PCR. Total RNA was isolated from 100 mg leaves using the Nucleospin® RNA Plant kit (Macherey-Nagel). The concentration was measured on a NanoDrop2000 (Thermo Scientific, USA). First strand cDNA was synthesized from 1 µg total RNA using the PrimeScript™ Reverse Transcriptase kit (Takara, Japan). qRT-PCR was performed on a Lightcycler 480 Real-Time PCR system (Roche, Switzerland). 3 µl of a reaction mixture comprising SYBR Green I Master (Roche, Switzerland), 10 µM each of forward and reverse primers and water, was added to 2 µL of a 10-fold diluted cDNA sample in a 384 well plate. The PCR program used was: 95 °C for 10 min, then 45 cycles of 95 °C for 15 s, 58 °C for 15 s and 72 °C for 15 s. At least three biological replicates were performed for each gene tested. Primers for all genes examined (Table S1) were designed using the Primer3Plus software. Profilin-1 (PRF1, AT2G19760) and Cyclophilin 5 (CYP5, AT2G29960) were used as reference genes for the normalization of gene expression levels.

Root growth measurements

All experiments were performed on WT Arabidopsis (Col-0). Seeds were surface sterilized in 70 % ethanol plus 0.05 % Triton X 100 and then in 100 % ethanol. Seeds were plated in square petri dishes containing Murashige and Skoog medium (MS^{-} ½) supplemented with 0.5 g/L MES-KOH pH 5.7, 0.8 % Plant Agar (Duchefa), and 1 % sucrose; stratified for 2 days at 4°C in the dark, and placed to grow vertically in a growth chamber under a long-day light period (16 h light/ 8 h dark) at 150 µmol m^{-2} s^{-1}. 0.5 µl or 5 µl of a 1.5 mM solution of β-CCA was added to 50 ml of growth medium (corresponding to 15 or 150 nM). Root growth was measured after 15 d.

Stomatal density and stomatal index

Epidermal fragments isolated from leaves of 4-week old Arabidopsis plants were attached to microscope coverslips by using silicone adhesive (Telesis 5) and incubated in a bathing solution (30 mM KCl, 10 mM Mes/Tris, pH 6). Stomata were analyzed by using an inverted microscope (Leica LMD-6000) with a 40x objective. Stomatal index was calculated by normalizing the number of stomata on the number of epidermal cells x 100.

Statistical analysis. All experiments were repeated at least two times, and the images represent typical examples. The values are represented as the means + standard deviation. The statistical significance was tested using Student's t-test (two-tailed, unequal variances). Sample size is reported in figure legends as number of plants per experiment.

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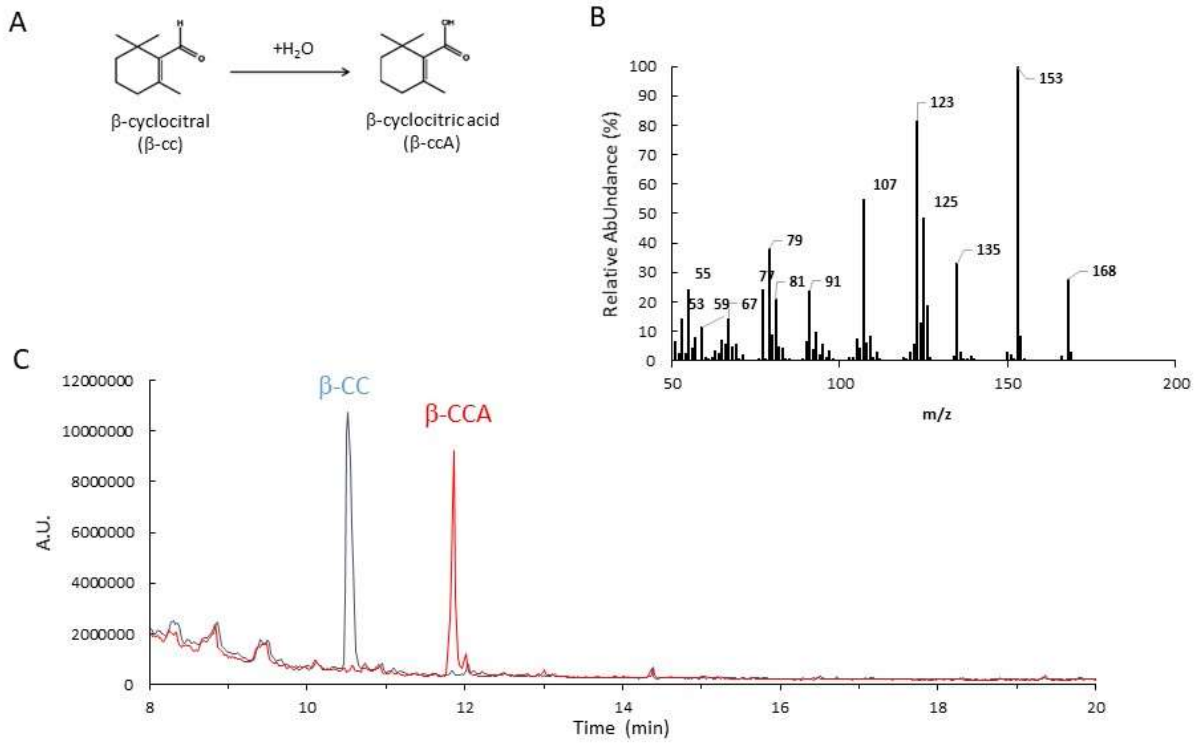

Figure S1. Conversion of β-cyclocitral (β-CC) to β-cyclocitric acid (β-CCA), related to Figure 1. (A) β-CC oxidizes to β-CCA in water. (B) Fragmentation plot of the peak corresponding to β-CCA. (C) GC-MS analysis of the oxidation of β-CC (m/z 137) into β-CCA (m/z 153). β-CC was injected in water and mixed in a closed bottle for 24 h and in an open bottle for 3 d. The graph shows GC-MS scan (TIC) at time 0 (in blue) and at time 4 d (in red). After 4 d, β-CCA is the only remarkable species in the solution with a concentration of about 1.5 mM.

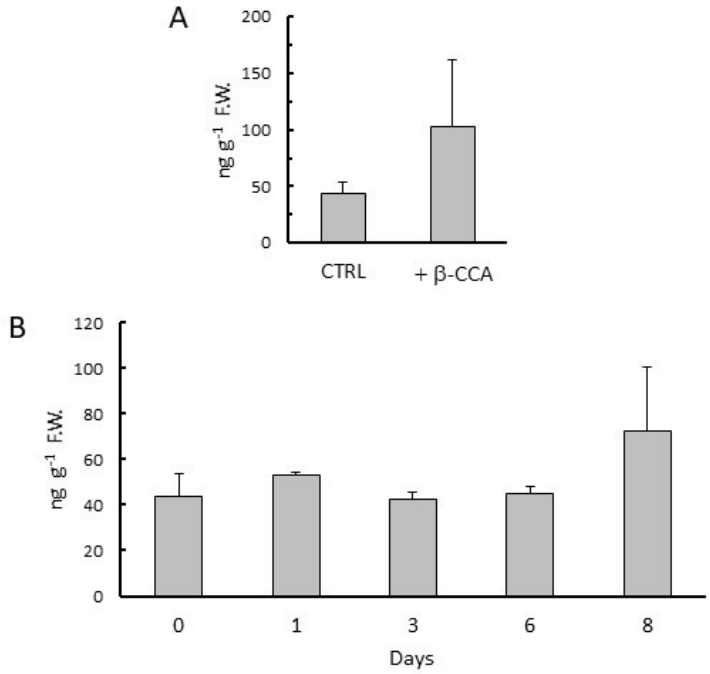

Figure S2. β-CC levels in leaves treated with β-CCA, related to Figure 1. A) Leaves directly treated with β-CCA (spray) (experiment of Fig. 1d). B) Leaves from plants watered with β-CCA (experiment of Fig. 1e). Data are mean values of 6 plants + SD.

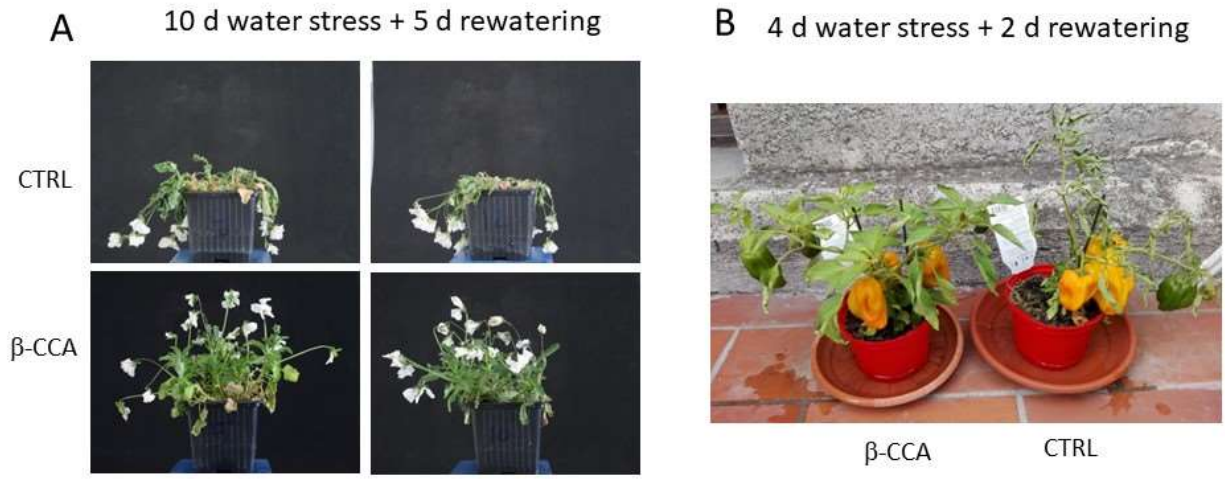

Figure S3. Protective effect of β-CCA against drought stress in different plant species, related to Figure 2. A) Pansy flower plants (*Viola bicolor*) exposed for 10 d to water stress by withdrawing watering followed by rewatering for 5 d. b) Pepper plants (*Capsicum annuum*) exposed for 4 d to water stress by withdrawing watering followed by 2 d of rewatering.

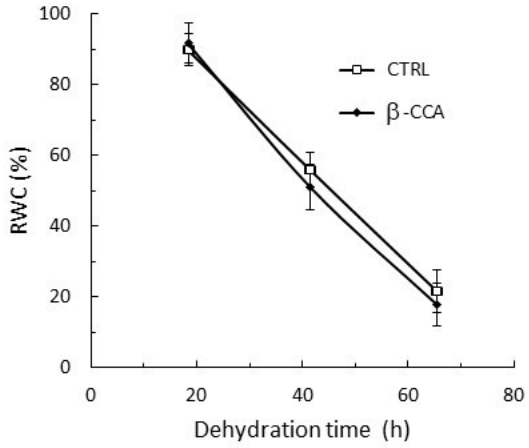

Figure S4. Drying rate of soil containing 0 or 1.5 mM β-CCA, related to Figure 2. Relative water content (RWC) of the soil was measured as: [(soil weight – soil dry weight)/(initial (watered) soil weight - soil dry weight)] x 100. Data are mean values of 4 experiments +SD.

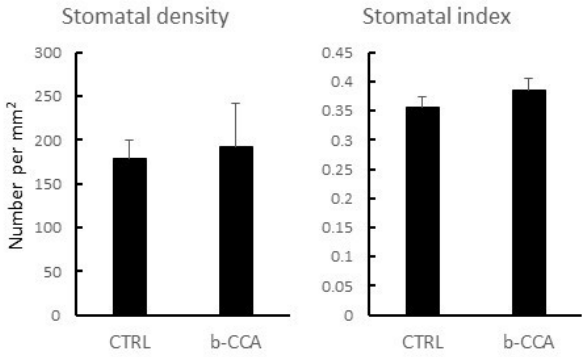

Figure S5. Stomatal density and stomatal index of leaves from β-CCA-treated and -untreated Arabidopsis plants, related to Figure 2. Leaves were taken 3 d after the β-CCA treatment (watering with 1.5 mM β-CCA). Data are mean values of minimum 20 microscopic images + SD.

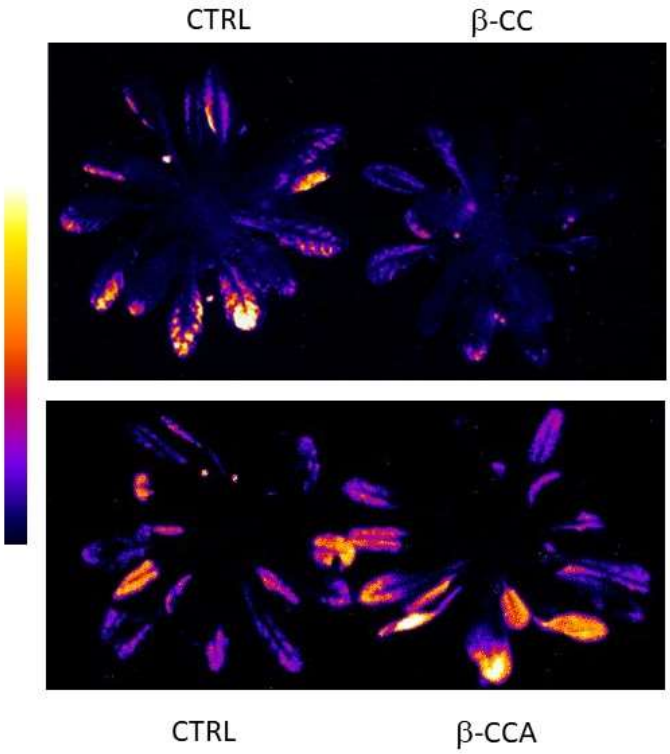

Figure S6. Effect of high light (1500 μmol photons m^{-2} s^{-1}) at low temperature (7°C) for 24 h on control (CTRL), β-CC-pretreated and β-CCA-pretreated Arabidopsis plants, related to Figure 5. Plants were exposed to volatile β-CC for 4 h. For the β-CCA treatment, plants were exposed high light and cold 48 h after watering the plants with 2.5 mM β-CCA-containing water. Lipid peroxidation was measured by autoluminescence imaging.

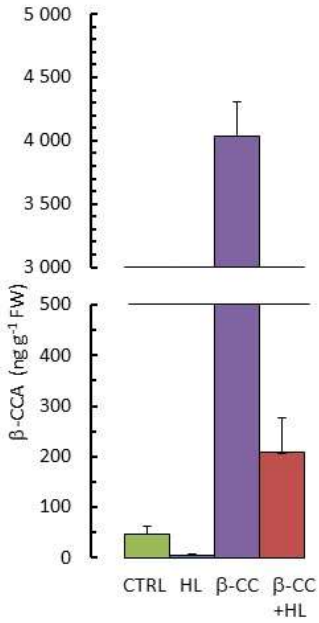

Figure S7. Effect of high light (HL, 1500 µmol photons m^{-2} s^{-1}) at low temperature (7°C) for 24 h on the β-CCA concentration in control (CTRL) and β-CC-pretreated Arabidopsis plants, related to Figure 5. Plants were exposed to volatile β-CC for 4 h. Data are mean values of 4 leaves + SD.

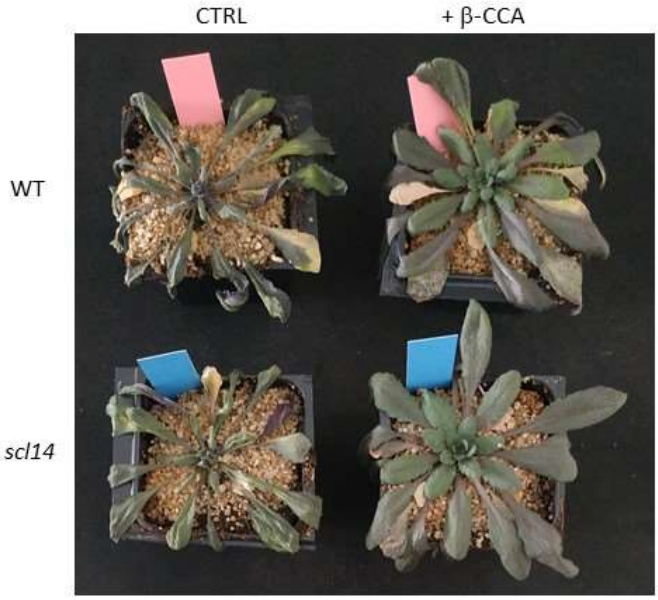

Figure S8. β-CCA protects the scl14 KO mutant against drought stress, related to Figure 6. Plants were treated with 1.5 mM β-CCA and water stress was imposed by withdrawing watering.

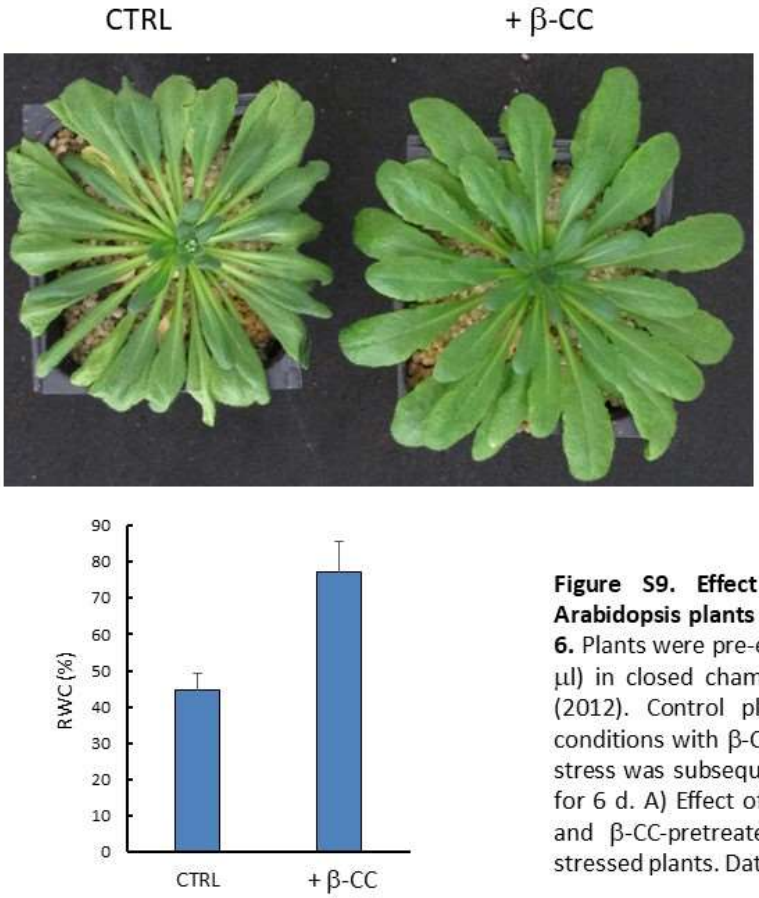

Figure S9. Effect of β-CC on the tolerance of Arabidopsis plants to drought stress, related to Figure 6. Plants were pre-exposed for 4 h to volatile β-CC (100 µl) in closed chambers, as described in Ramel et al. (2012). Control plants were exposed to the same conditions with β-CC being replaced by water. Drought stress was subsequently induced by stopping watering for 6 d. A) Effect of water deprivation on control (crtl) and β-CC-pretreated plants. B) RWC of the water-stressed plants. Data are mean values of 6 leaves + SD.

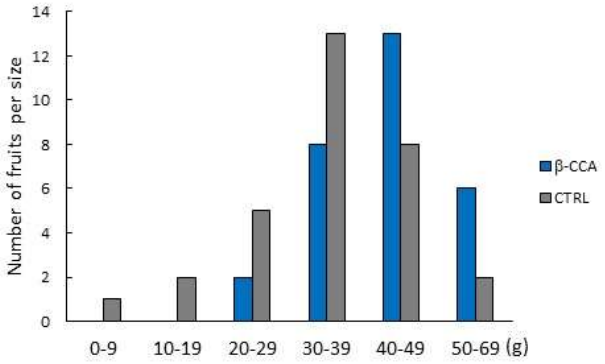

Figure S10. Number of fruit per size class (g) of the experiment used for calculating the average weight of fruits, related to Figure 7.

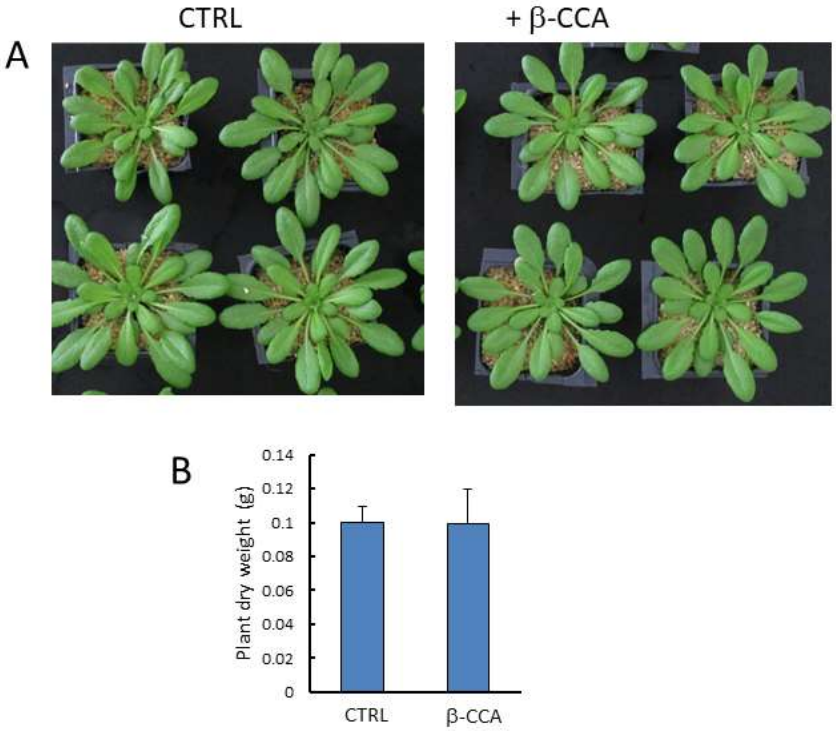

Figure S11. Long-term exposure of Arabidopsis plants to β-CCA had no effect on the growth rate, related to Figure 2 and Figure 3. Plants aged 10 d were watered for 30 d with water containing β-CCA in order to provide plants with 6 mg β-CCA per plant and per week. A) picture of the plants and B) shoot dry weight at the end of the experiment. Data are mean values of 6 plants + SD.

Table S1. Primer list for qRT-PCR, related to Figure 1, Figure 2, Figure 5 and Figure 6.

AT5G61820	for: TGGGTTGATTGGGACAGCTC rev: TCACCTCGTATTCCACATGGC
AT5G63790	for: AGCTTCCAGAAATGGCGTTGT rev: AATAACCGGTTCCAGCTGCC
AT3G04000	for: CGCATTATCAGTGTGAACACAAGAG rev: TTTGAACCAGTGATGTTGAGAGGAG
AT3G28580	for: ATGGCGATGATGGGTCAGTT rev: GGGTAGAAGCGACCGAAGAG
AT5G16970	for: CCATGGTAAGAATGTTGGGAAACAA rev: AAAACAAAAACCACCCACACAAACT
AT4G27410	for: CCACCAGGTTTTAGATTTTATCCG rev : CAAAGCCTTACTTGGCAAATCC
AT5G05410	for: TCGTCCCCTATAGATTGTGTTGT rev : GCCACAGTAGTACCGTCACC
AT1G01720	for: CCATGGGAGCTTCCTGGTTT rev : ACGCGAACCGTTGGGATATT
AT5G52300	for : CACAGCTTTGGAAAATGGAGTCA rev : CATGATGCTCTTCTTCTTCTGGAT
AT5G52310	for : GCACCAGGCGTAACAGGTAA rev : TCGGAAGACACGACAGGAAA
AT5G25610	for : CCAACTCCCAAAAATGGCGA rev : CGCAATCGCCACTACCATGA
AT1G42990	for : GAAGGAGACGATGATGCTGTGGCT rev : AGCAGGGAACCCAACAGCAGACT (spliced)
CYP5	for: CTGGACCAGGTGTACTTTCAATGG rev: AAACACCACATGCCTTCCATCTAAC
PRF1	for: AGAGCGCCAAATTTCCTCAG

rev: CCTCCAGGTCCCTTCTTCC