Plant Biotechnology Reports (2022) 16:265–282
https://doi.org/10.1007/s11816-021-00733-x

Online ISSN 1863-5474
Print ISSN 1863-5466

REVIEW

Eligible strategies of drought response to improve drought resistance in woody crops: a mini-review

Elia Carraro[1] · **Antonino Di Iorio**[1]

Received: 19 September 2021 / Revised: 25 November 2021 / Accepted: 10 December 2021 / Published online: 13 January 2022

Abstract

Drought is the main abiotic stress that negatively affects the crop yield. Due to the rapid climate change, actual plant defence mechanisms may be less effective against increased drought stress and other related or co-occurring abiotic stresses such as salt and high temperature. Thus, genetic engineering approaches may be an important tool for improving drought tolerance in crops. This mini-review focuses on the responses to drought stress of the woody crop species *Olea europaea* and *Citrus* sp., selecting in particular five main strategies adopted by plants in response to drought stress: aquaporin (AQPs) expression, antioxidant activity, ABA signalling, and trehalose and proline accumulation. Transgenic studies on both the herbaceous *Arabidopsis* and woody *Populus* plant models showed an improvement in drought resistance with increasing expression of these drought-inducible genes. Outcomes from the present study suggest the overexpression of the gene families associated with AQPs and ABA biosynthesis, mainly involved in regulating water transport and in preventing water loss, respectively, as candidate targets for improving drought resistance; antioxidants-, trehalose- and proline-related genes remain valid candidates for resistance to a wider spectrum of abiotic stressors, including drought. However, the contribution of an increased stiffness of the modulus elasticity of leaf parenchyma cell walls to the rapid recovery of leaf water potential, delaying by this way the stress onset, is not a secondary aspect of the transgenic optimization, in particular for *Olea* cultivars.

Keywords Woody crops · Drought tolerance · Drought avoidance · Citrus cultivars · Olea cultivars · Genetic engineering

Introduction

Due to climate change, polluting activities and the continuously growing world population, water availability and water quality have been lowering. On the other hand, the agricultural water demand, which accounts for 70% of water use worldwide (OECD), is incessantly increasing. Lack of water can have detrimental effects on plants, narrowing crop yield and productivity and causing huge economic losses. Therefore, water scarcity has been and still will be considered as an urgent global and environmental problem. Drought is the main abiotic stress that promotes an imbalance between root water uptake and water loss via transpiration which results in plant dehydration. Furthermore, some other stresses like high temperature are usually co-incidental to drought stress.

During their evolution, plants have developed four drought resistance mechanisms that allow them to overcome water deficit: drought avoidance (DA), drought tolerance (DT), drought escape (DE) and drought recovery (DR) (Lawlor 2013; Fang and Xiong 2015). DA is the ability of plants under mild or moderate drought stress conditions to store as much water as possible and to sustain basal metabolic processes that allow them to survive. For this purpose, plants adopt the following strategies that permit them to reduce water loss and enhance water uptake: rapid stomatal closure, leaf rolling (reduces the leaf area exposed to incident radiation), wax accumulation on cell surface (reflects the sunlight and prevents excessive transpiration), increased root/shoot ratio, rooting depth and enhanced water storage capabilities. DT is the ability of plants under severe drought stress conditions to endure low tissue water content and to maintain a certain level of physiological activities by repairing stress damages, mainly oxidative and osmotic damages. DE is the ability of plants to complete their life cycle before the onset of drought stress, thereby plants do not experience drought stress. DR is the ability of plants to recover from a

✉ Antonino Di Iorio
antonino.diiorio@uninsubria.it

[1] Department of Biotechnology and Life Sciences, Università Degli Studi Dell'Insubria, Via Jean Henry Dunant, 3, 21100 Varese, Italy

dehydrated status after being exposed to a prolonged drought stress.

Each species can perform more than one of the mechanisms described above depending on the developmental stage, making it rather difficult to comprehensively and accurately evaluate the overall drought resistance strategy adopted. Nevertheless, for more than two decades, the body of literature on both morphological traits and structural, physiological, biochemical, and molecular regulation of above- and belowground organs in response to drought stress is consistently increased. In recent years, many efforts had been addressed at elucidating the biochemical, genetic, and signalling networks involved in plant drought responses; however, the underlying sophisticated mechanisms that differentiate resistance from susceptibility within a species, especially for crops, remain largely unclear.

Citrus and *Olea* are genera of crop trees growing in tropical and Mediterranean environments where drought periods are common. Although during the evolution they have adapted to several abiotic stresses, water scarcity is still a threatening factor that negatively affects their growth, productivity, and fruit quality. Citrus and olive global production, as well as the majority of crops of agronomic interest, have grown in the last decades (ec.europa.eu; FAO); thus, in the context of climate change predictions of water scarcity, it is important to develop water-saving and drought-resistant crops. The outcome of the genomes of olive in 2016 (Cruz et al. 2016), with about 1.38 Gb (G bases) total length, and of citrus in 2019 (CGD, https://www.citrusgenomedb.org) and 2020 (the CitGVD database, Li et al. 2020) opened new opportunities in the study of the different molecular traits and phenotypic variations within these species.

The response of plants to drought stress is a complex process involving many genes and signalling pathways, but it has been proven that multiple mechanisms are involved (Lawlor 2013). Moreover, multi-gene transformation strategy that combines several major functional or regulatory genes or a series of genes in a signalling cascade may be undoubtedly more reasonable or promising than single-gene transformation for improving drought resistance in plants (Fang et al. 2015). The most interesting and promising genetic strategies addressed by this review are (a) the regulation of aquaporin (AQP) expression, (b) antioxidant activities, (c) ABA signalling, and (d) trehalose and (e) proline accumulations. Furthermore, the selection of drought-resistant plants requires experimental settings that control the water status of plants and assess the effects of water deficits on physiological processes such as growth, photosynthesis, dry matter production, and water loss (Lawlor 2013). Therefore, the measurement of at least the water status in terms of the energetics of water (Ψ and π), the water content (RWC), and the duration of the drought period (or soil drying) are very important in evaluating which strategy to optimize.

This review addresses the progress on the selected genetic actors for improvement of drought resistance in the woody crops *Citrus* and *Olea*, and analyses which of them better depicts the species-specific response, if any, in wild relatives and elite cultivars. To this aim, a small database was compiled from the literature which also considers the leaf water potential and the duration of drought treatment.

Regulation of AQP expression

AQPs are transmembrane proteins belonging to the major intrinsic proteins (MIPs) superfamily; they are involved in the symplastic transport of water and other small neutral solutes, mainly CO_2. According to their subcellular localization and function, plant AQPs are classified into five subfamilies: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD 26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs) and unclassified X intrinsic proteins (XIPs) (Rodríguez-Gamir et al. 2011). Each subfamily can be further divided into different subgroups. For instance, PIP subfamily encompasses PIP1 and PIP2 subgroups. Eventually, each subgroup is further divided into different isoforms such as PIP1;1, PIP1;2, PIP2;1 and so on (Afzal et al. 2016). However, some structural domains are highly conserved among the different subfamilies such as the NPA motif (Asn-Pro-Ala) which confers selectivity for water molecules (Wei et al. 2019). As intrinsic structural membrane proteins, activation and deactivation of AQPs are mediated by post-transcriptional regulation which involves phosphorylation and variation of cytosolic pH and Ca^{2+} content (Zargar et al. 2017).

The number of AQPs varies between plants; for example, in *Citrus*, the number of AQPs identified (34) is lower than in Poplar (55) (Wei et al. 2019). PIPs and TIPs are mainly involved in water transport, while NIPs, SIPs and XIPs are found to have higher solute transport activity (Zargar et al. 2017). Regarding drought tolerance, PIPs are probably more significant than TIPs in regulating root water uptake because the plasma membrane is much less permeable to water than the tonoplast (Secchi and Zwieniecki 2014). Hence, because of their involvement in water transport, PIPs are considered as prime targets for the improvement in drought stress tolerance.

It is difficult to provide a general expression pattern of the *AQP* genes in response to drought stress. Studies of *PIP* genes expression to drought stress showed variable responses of up, down or no regulation at all, even among the same plant species (Afzal et al. 2016). In general, plants respond to drought stress by downregulating PIP gene expression, especially in *Citrus* and *Olea oleaster* trees.

For example, in three different citrus rootstocks exposed to drought (Rodríguez-Gamir et al. 2011), *Poncirus trifoliata* (PT), *Cleopatra mandarin* (CM) and the hybrid 030115 (CMxPT), all grafted on the *Valencia Late* (citrus) tree, the *PIP* gene expression levels were lower in CM and the hybrid, whereas no significant changes occurred on PT. Consistently, a more recent study proved that in roots of two *Citrus* trees exposed to drought, *Sanhuhongju* (HJ) and *Sanhuhuahong* (HH), the majority of *CsPIPs* (*Citrus* PIPs) and *CsTIPs* genes were downregulated (Wei et al. 2019). Similarly, in the shoots of *Olea europaea*, *OePIP2.1* (*O. europaea* PIPs) aquaporin gene exhibited a lower expression under drought stress condition (Secchi et al. 2007). The downregulation of PIP genes during drought stress would reduce cell water permeability by both promoting cellular water conservation (Secchi et al. 2007) and avoiding the reverse water flow into soil (Wei et al. 2019). Moreover, *PIPs* downregulation could indirectly promote the lowering of stomatal conductance by reducing the water flow to the leaves (Zargar et al. 2017). In particular, PIP1 aquaporin appeared to play a key role in facilitating PIP2 water transport, but not vice versa. Indeed, if expressed in *Xenopus* oocytes, the membrane permeability to water was much higher under the co-expression of both PIPs rather than PIP2 alone (Secchi and Zwieniecki 2014; Rodríguez-Gamir et al. 2011).

PIP1 could have an important role also in xylem recovery from embolism as demonstrated in a transgenic poplar tree (*Populus alba* × *Populus tremula*) characterized by the strong downregulation of multiple PIP1 isoforms (Secchi et al. 2014). In this study, transgenic plants exposed to drought stress were more subjected to embolism and had a reduced capacity to restore xylem conductance during recovery. Therefore, *AQPs* upregulation at the end of the drought stress period may promote a fast recovery of leaf water status. According to this hypothesis, in *O. europaea* leaves subjected to drought stress, *OePIP1.1* and *OePIP2.1* genes exhibited an increased expression at the beginning of recovery period (Perez-Martin et al. 2014; Araújo et al. 2019). On the other hand, these olive plants showed an *OePIP1.1* upregulation also during the first days of stress, suggesting a strategy to initially maintain leaf turgor in a moment of low water availability in soil. Overexpressing AQP genes can generally confer a better resistance to drought and osmotic damages, which often come together (Afzal et al. 2016). The advent of *Agrobacterium tumefaciens*-mediated genetic transformation made possible the insertion of AQPs gene sequences of typical drought-tolerant plants in drought-sensitive species. In a transgenic *Arabidopsis*, the *ScPIP1* drought-induced gene of the desert plant Jojoba (*Simmondsia chinensis*) was inserted under the control of the 35S promoter (Wang et al. 2019). After exposure to different periods of drought, transgenic lines overexpressing the Jojoba *ScPIP1* exhibited longer root lengths, better growth status, higher survival rates, higher proline contents and reduced malondialdehyde than the wild type, resulting in a plant with enhanced resistance to drought, osmotic and oxidative stresses.

The differences between the various expression patterns of PIPs depend on the aquaporin isoform, tissue, stress level, plant species and many other factors. However, in many studies, a general downregulation is observed when the plant is exposed to prolonged drought stress, primarily in *Citrus* and *Olea* trees. This suggests that the upregulation of AQPs during the first stage of drought stress, particularly in roots, may help to absorb as much water as possible from the soil to maintain initial leaf turgor. Later on, during recovery, higher PIP expression at the shoot level would enhance the xylem refilling capacity of parenchyma cells to avoid embolism damage.

Antioxidant defence mechanism

One of the major consequences of drought and environmental stresses, in general, is the overaccumulation of reactive oxygen species (ROS) that cause oxidative damages in plants. ROS are unstable molecules, ions and free radicals containing oxygen; they easily react with other molecules in a cell, resulting in being deleterious when present in high concentration. Typical ROS are the superoxide anion ($O_2\bullet^-$), hydrogen peroxide (H_2O_2), hydroxyl radical (HO•) and singlet oxygen ($^1O_2$). Under normal growth conditions, ROS are formed as a by-product of the aerobic metabolism in chloroplasts, mitochondria and peroxisomes, where there is an important flux of electrons due to the high metabolic activity of these organelles. A minimum amount is essential for the correct functioning of the cell, as they are rapidly detoxified by enzymatic and non-enzymatic antioxidants naturally occurring in plants (Zandalinas et al. 2017). Under drought stress conditions, the accumulation of ROS overcomes the detoxifying capacity of the antioxidant machinery, and the cell undergoes a state of oxidative stress that damages organelles and causes metabolic imbalances (Caverzan et al. 2016). Furthermore, ROS are produced also during biotic stresses acting as toxic molecules against pathogens (Huang et al. 2019).

During stress response, ROS accumulation leads to the upregulation of multiple genes encoding for antioxidant enzymes, to provide a better tolerance against oxidative stress (Sofo et al. 2005). Membrane lipid peroxidation is one of the consequences of ROS accumulation and leads to malondialdehyde (MDA) production, whose content is directly proportional to the severity of oxidative stress (Hussain et al. 2018). In this context, during the evolution, plants have developed enzymatic and non-enzymatic antioxidant

defence mechanisms to mitigate the deleterious effects of oxidative stress (Denaxa et al. 2020).

Among the enzymatic systems, the most important are: superoxide dismutase (SOD), which catalyses the reaction from $O_2\bullet^-$ to H_2O_2; catalase (CAT), mainly localized into peroxisomes, reduces H_2O_2 to 2 H_2O; peroxidase (POD), both involved in scavenging H_2O_2 in chloroplast and enhances growth and development of the plant; ascorbate peroxidase (APX), which catalyses H_2O_2 detoxification through the ascorbate–glutathione (AsA–GSH) cycle (Fig. 1); glutathione reductase (GR), which helps in maintaining high levels of reduced glutathione (GSH) in the AsA–GSH cycle by reducing oxidized glutathione (GSSG) to GSH in a NADPH-dependent reaction (Caverzan et al. 2016). The preservation of a favourable GSH/GSSG ratio has been frequently found in highly drought-tolerant plants (Zandalinas et al. 2017).

The non-enzymatic systems (Fig. 1) encompass: ascorbate (AsA), which donates one electron to APX to detoxify H_2O_2; reduced glutathione (GSH), which donates one electron to facilitate AsA regeneration by the reduction of ROS as well as tocopherol, carotenoids, and phenolic compounds (Caverzan et al. 2016). Thus, along with SOD and CAT activity, the AsA–GSH cycle is an important pathway involved in chloroplast, mitochondria, peroxisomes and cytosol ROS scavenging.

Contrarily to AQPs, enzymatic and non-enzymatic antioxidants have a uniform and well-known expression pattern that, under drought stress conditions, faces a general upregulation to counteract the oxidative status within the cell.

Experiments with *Citrus* sp. and *O. europaea* revealed that enhanced drought tolerance is correlated to a better functioning of the antioxidant machinery. In a work performed on six one-year-old *Citrus* rootstocks (*Volkameriana lemon* (V_1), *Brazilian sour orange* (V_2), *Carrizo citrange* (V_3), *Eureka lemon* (V_4), *Gada dahi* (V_5), and *Rangpur lime* (V_6)) exposed to 24 days of progressively soil drying (Hussain et al. 2018), the activity of the three major ROS-scavenging enzymes (SOD, CAT and POD) along with other attributes like H_2O_2, MDA and total soluble protein (TSP) concentrations have been analysed in leaves and roots to better understand the relationship between the antioxidant defence mechanism and the plant physiological status. The expression patterns of antioxidant enzymes were more frequent in leaves (Fig. 2) than in roots (data not shown). SOD and CAT reached the peak after 18 days of drought, in a moment of moderate stress, for slightly decreasing on day 24, although remaining always higher than control (Fig. 2a, b). Differently, POD activity increased till the 24th day of stress (in a moment of severe drought stress for the plants). SOD and CAT were the antioxidant enzymes with the highest activity, highlighting a leading role as ROS scavenger triggered by oxidative stress. TSP content followed the same pattern of SOD and CAT and its increase may be due to the expression of new stress proteins that activate the antioxidant defence mechanism. After 24 days, stressed plants had likewise maximum H_2O_2 and MDA content compared to control plants and, for both parameters, the values were higher in leaves than in roots (Fig. 3). These results make the chloroplasts the main ROS producers, due to their sustained electron flow (Sofo et al. 2005). Among the different species studied, *Carrizo citrange* showed enhanced drought tolerance along with higher antioxidant activity and lower MDA and H_2O_2 content. Thus, this study confirmed the positive correlation between drought tolerance and the correct functioning of the antioxidant machinery.

Similar results have been observed when two *Citrus* cultivars (*Carrizo citrange* and *Cleopatra mandarin*) were

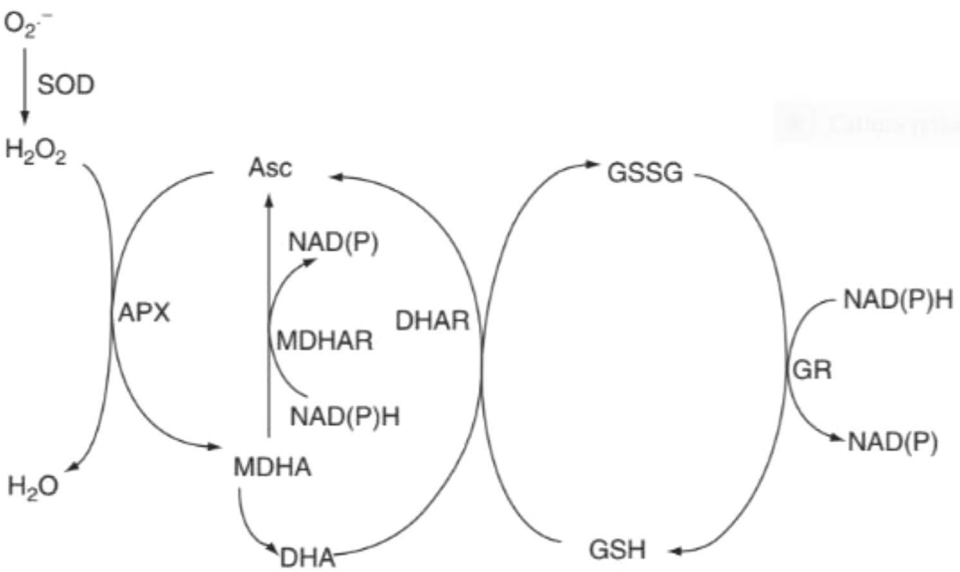

Fig. 1 The scheme of AsA–GSH cycle. *Asc* ascorbate, *APX* ascorbate peroxidase, *DHA* dehydroascorbate, *DHAR* dehydroascorbate reductase, *MDHA* monodehydroascorbate, *GR* glutathione reductase, *GSH* reduced glutathione, *GSSG* oxidized glutathione (from Latowski et al. 2010)

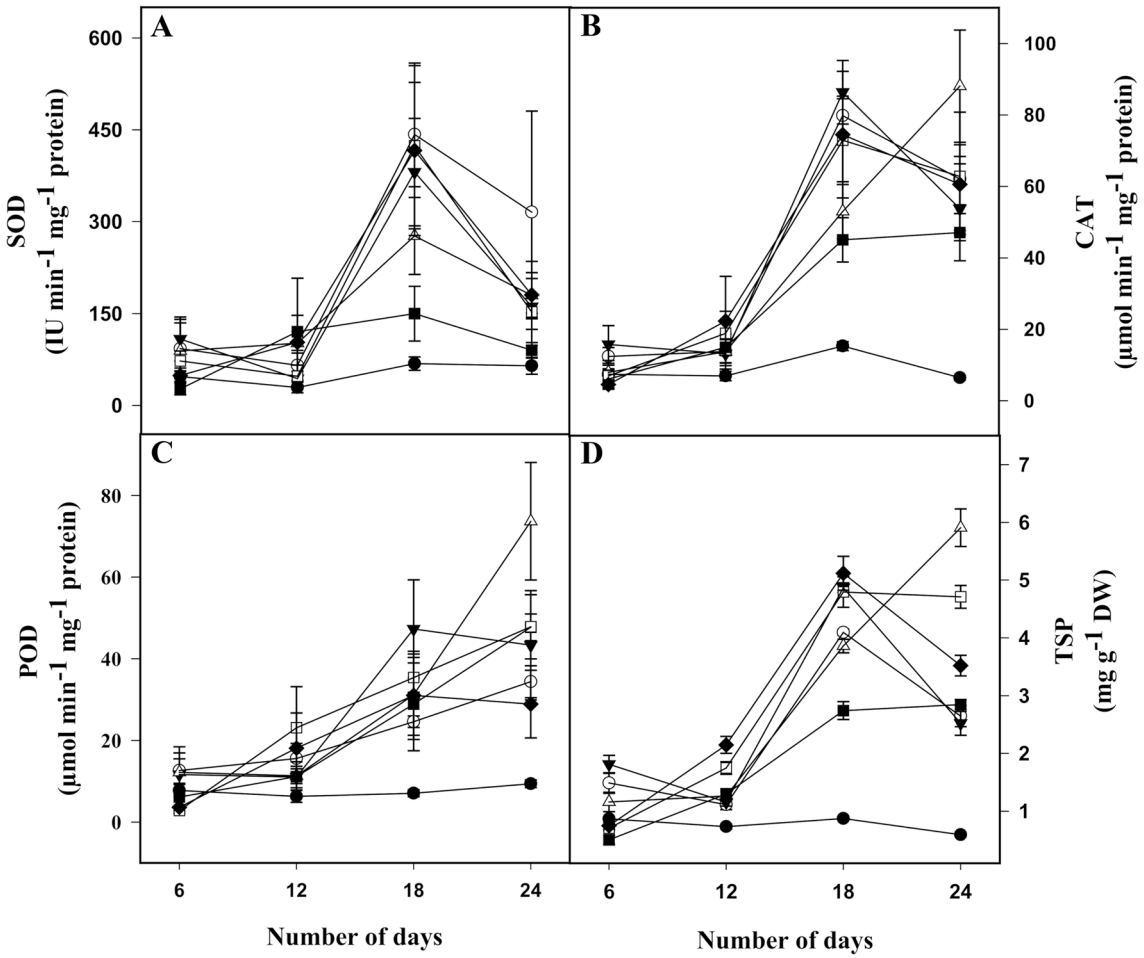

Fig. 2 **A** SOD; **B** CAT; **C** POD; **D** TSP in leaves of six citrus rootstocks during 24 days of drought stress. For each date, the control represents the mean value of six rootstocks. Values are mean ± SE at $p < 0.05$ ($n = 3$). Symbols presented in graphs correspond to: filled circles: control; open circles: volkameriana lemon; inverted filled triangles: Brazilian sour orange; upright open triangles: Carrizo citrange; filled squares: Eureka lemon; open squares: Gada dahi; filled diamonds: Rangpur lime (from Hussain et al. 2018)

exposed to the combination of heat and drought stress (Zandalinas et al. 2017). After imposing 7 days of high temperatures (40 °C), a group of plants was exposed to a 24 h water stress period by transplanting to dry perlite. Again, *Carrizo citrange* showed a more drought-tolerant phenotype along with a higher activity of SOD, CAT, APX and GR compared to *Cleopatra mandarin*. In addition, AsA and GSH content increased in both plants when stressed, especially when subjected to both heat stress + drought stress; this suggests that the non-enzymatic antioxidants may be more important in combined stress conditions or when the stress pressure is stronger. *Cleopatra mandarin*, the less drought-tolerant cultivar, showed higher MDA content and a lower GSH/GSSG ratio caused by GSSG accumulation. Thus, a more favourable GSH/GSSG ratio (such as in *Carrizo citrange*) allows a better tolerance to oxidative stress and, consequently, to drought stress.

Olive trees subjected to drought exhibited a similar regulation of the antioxidant machinery, as reported in the study of Sofo et al. (2005). In this experiment, 2-year-old *Coratina* (*O. europaea*) plants underwent 20 days of drought stress. A general upregulation of SOD, CAT, POD and APX was observed in response to drought-induced oxidative damages, and similarly to citrus, these enzymes had the highest activity in leaves.

The antioxidant defence mechanism is ubiquitous in all plants. A transgenic approach with the *Arabidopsis*-defective mutant for the ascorbate peroxidase 1 (APX1) enzyme resulted in higher sensitivity to combined drought and heat stress (Zandalinas et al. 2017). In contrast, *Arabidopsis* plants overexpressing one or more antioxidant genes showed an enhanced antioxidant capacity and were more resistant to a broad range of abiotic stresses (Caverzan et al. 2016).

Based on these results, pursuing the upregulation of antioxidants in leaves, especially SOD, CAT and GR which are

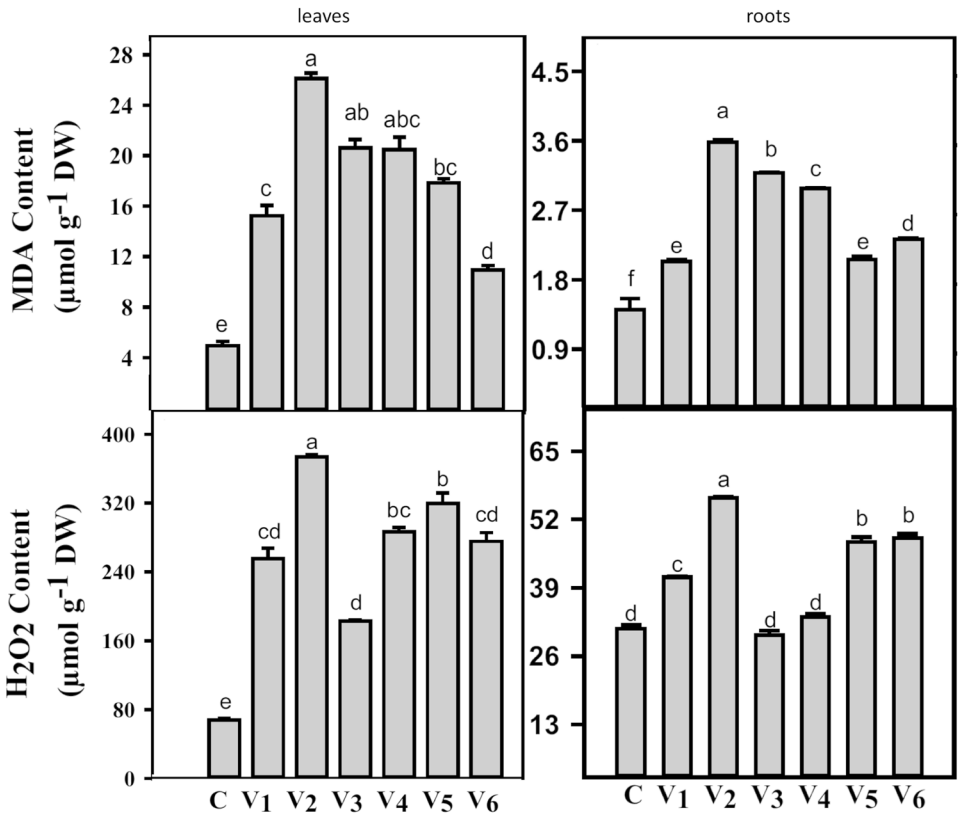

Fig. 3 MDA and H_2O_2 concentrations at the 24th day in leaves and roots (columns) of six citrus rootstocks under drought stress. Control values represent the mean value of six rootstocks. Values are mean ± SE at $p < 0.05$ ($n = 3$). C control, V1 volkameriana lemon, V2 Brazilian sour orange, V3 Carrizo citrange, V4 Eureka lemon, V5 Gada dahi, V6 Rangpur lime (adapted from Hussain et al. 2018)

involved in the first response against ROS, and the maintenance of a favourable GSH/GSSG ratio could reasonably improve drought tolerance in crops.

Trehalose accumulation

Trehalose is a non-reducing disaccharide formed by two α-glucose units linked in a 1,1-glycosidic bond. It is widely spread in lower organisms such as bacteria, yeasts, fungi, as well as in plants, insects, and many other invertebrates. Trehalose has been found not only to act as a source of carbon molecules, but mainly as a protective compound in response to abiotic stresses. Indeed, in lower organisms, it accumulates under heat, drought or salt stress to preserve the membranes from desiccation damages and to promote osmotic adjustment (Iordachescu and Imai 2008). Particularly, under limited water supply, trehalose accumulation is necessary to prevent the transformation of the phospholipid bilayer membrane from the liquid crystal state to the solid state, and stabilize the structure of proteins, nucleic acids, and other biomolecules (Fang et al. 2015). This is possible thanks to the trehalose unique feature of reversible water absorption capacity that protects biological molecules from desiccation-induced damage (Penna 2003). During severe dehydration, water molecules dissociate from the polar residues of cell macromolecules, but are replaced by sugars, mainly trehalose, which form hydrogen bonds with the residues and help them at stabilizing their structure and maintaining their activity. Even if trehalose seems to be extremely useful in stress resistance, plants (except resurrection plants) exhibit low increase in its content when exposed to drought stress compared to microorganisms (Penna 2003). This behaviour might indicate that trehalose does not have a direct role in plant's protection from abiotic stresses, but may act as a modulator that triggers other stress-responsive mechanisms (Santana-Vieira et al. 2016; Iordachescu and Imai 2008). In fact, in transgenic plants constitutively expressing microbial trehalose biosynthetic genes, trehalose levels were higher compared to WT, but still lower than expected; however, transgenic lines exhibited a better tolerance to drought than the WT (Iordachescu and Imai 2008; Lin et al. 2019). This proves that there is a positive correlation between trehalose accumulation and a better resistance to drought stress. In addition, low trehalose content may be caused by the enzyme trehalase which is ubiquitously present in plants and promotes trehalose degradation in its two glucose monomers (Penna 2003). Thus, it may be possible to increase trehalose production in plants by reducing trehalase activity.

In plants, trehalose biosynthesis consists of two consecutive reactions:

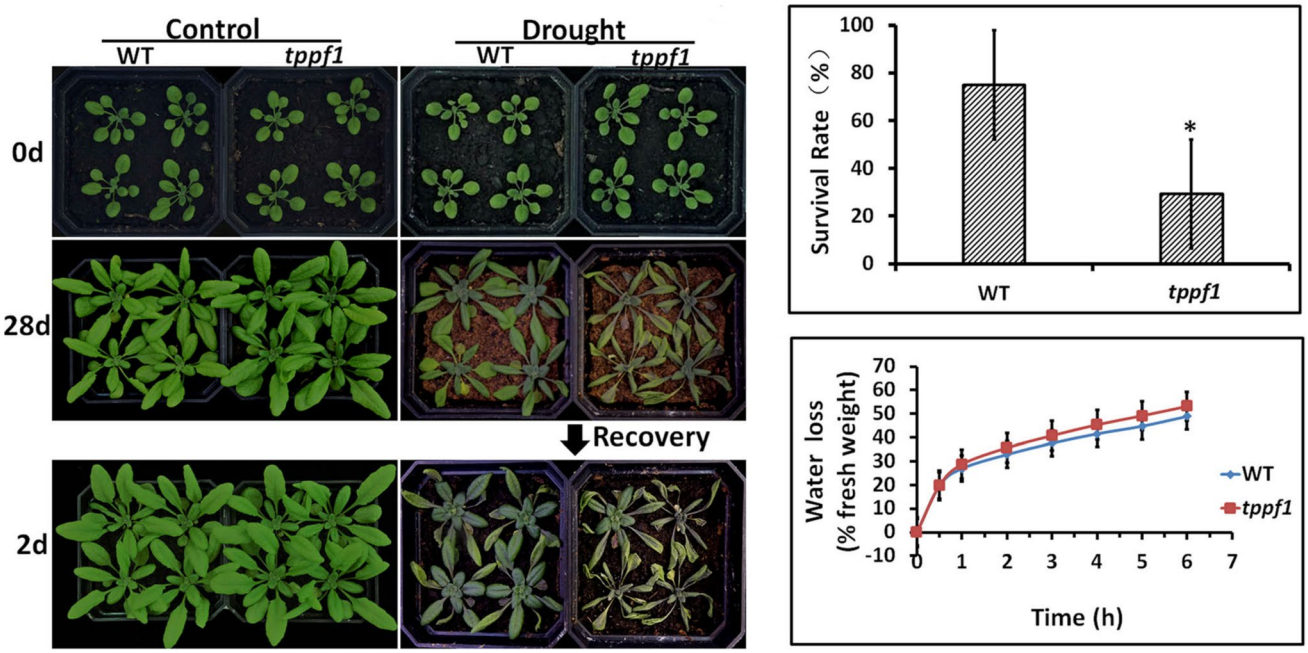

Fig. 4 *tppf1* mutant is more sensitive to drought stress than the WT *Arabidopsis thaliana*. Pictures show the phenotypes of WT and defective plants exposed to watering and drought conditions. Top right chart compares drought-stressed plants' survival rates 4 days after rewatering. Bottom right chart compares water loss from detached leaves in both WT and mutant lines (from Lin et al. 2019)

(1) Trehalose-6-phosphate synthase (TPS) catalyses the reaction between UDP-glucose and glucose-6-phosphate to obtain trehalose-6-phosphate.
(2) Trehalose-6-phosphate phosphatase (TPP) catalyses the dephosphorylation of the product of the first reaction to obtain trehalose.

In the last decades, important works of genome sequencing allowed to identify 11 TPSs (from *AtTPS1* to *AtTPS11*) and 10 TPPs (from *AtTPPA* to *AtTPPJ*) genes in *Arabidopsis thaliana*. Later studies with gain-of-function and loss-of-function mutants have been crucial for a better understanding of the role of trehalose in plants. For example, the *Arabidopsis tps1* knockout mutant was found to be embryo lethal (Iordachescu and Imai 2008); this finding proved that the trehalose pathway is vital in plants and could have a role in the early stages of development. On the other hand, *Arabidopsis* mutants overexpressing the *AtTPS1* gene resulted in more drought-tolerant lines but with a very low detectable increase in trehalose content.

Trehalose biosynthesis has been found to be involved in multiple abiotic stresses, but it also plays an important role in vegetative growth. Depending on environmental conditions, TPSs and TPPs are differentially expressed in roots and shoots (Lin et al. 2019). An *in silico* analysis was carried out on *Arabidopsis* TPSs and TPPs genes to study their expression pattern in response to different abiotic stresses such as cold, osmotic, salt, drought, oxidative, genotoxic, UV-B, wounding and heat (Iordachescu and Imai 2008). This study showed that trehalose biosynthesis genes are differentially upregulated or downregulated depending on the type of stress affecting the plant.

In this context, a study was performed on the overexpression of *AtTPPF*, a member of the *Arabidopsis* TPP gene family highly induced under drought stress (Lin et al. 2019). The study consisted in the creation of a loss-of-function and an overexpressing *Arabidopsis* mutant for the *AtTPPF* gene obtained by T-DNA insertion. Both lines were exposed to 4 weeks of drought, followed by 2 days of recovery. The defective mutant resulted in a more drought-sensitive plant, although phenotypically similar to the WT under control conditions (Fig. 4). In contrast, the overexpressing mutants, driven by the *CaMV 35S* constitutive promoter, exhibited an enhanced drought tolerance compared to the WT. In particular, three homozygous overexpressing lines (OE5, OE6 and OE9) with different *AtTPPF* expression levels were selected, and their degree of drought tolerance was directly proportional to the transcript levels of *AtTPPF* (Fig. 5). No significant changes were observed between WT and the loss-of-function mutants. Under drought stress, OE9 line exhibited lower H_2O_2 content in the shoot apical meristem (SAM), where *AtTPPF* is primarily expressed, compared to WT and loss-of-function plants; this suggests that trehalose may have a role in the antioxidant defence mechanism. Moreover, soluble sugar content, especially sucrose, was found to be higher in the OE9 mutant. Sugar accumulation

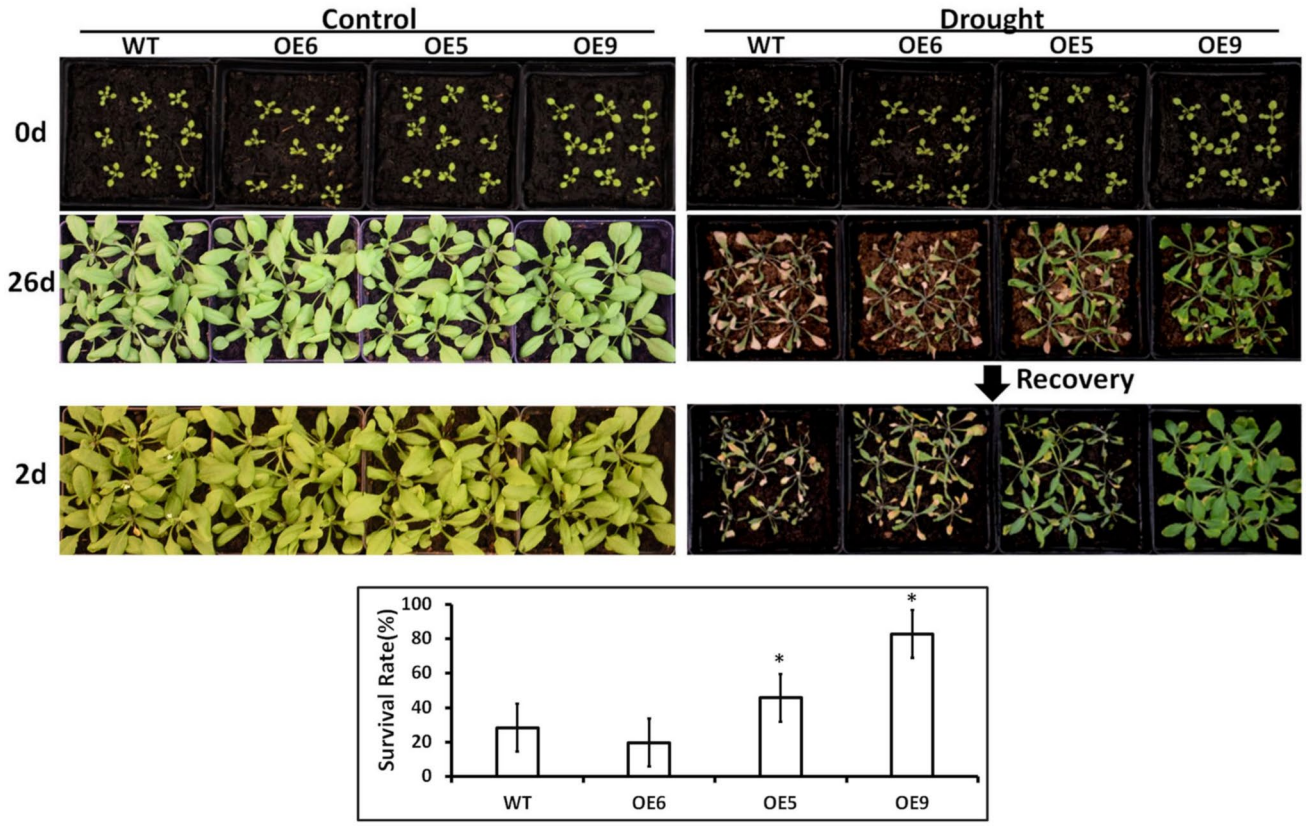

Fig. 5 *AtTPPF* overexpression mutants are more drought tolerant than WT *Arabidopsis thaliana*. Pictures show the phenotypes of WT and three different overexpressing lines exposed to watering and drought conditions. The bottom chart compares drought-stressed plants' survival rates 2 days after rewatering (adapted from Lin et al. 2019)

in the cytoplasm is a typical drought-tolerant mechanism, as it reduces cell osmotic potential to facilitate cell water retention, a procedure named "osmotic adjustment" (OA) (Fang et al. 2015). Hence, the upregulation of trehalose biosynthetic genes positively affects drought tolerance in plants, probably by triggering other genes and/or acting on ROS scavenging and osmotic regulation. According to this hypothesis, a transcriptome analysis of the WT and the *AtTPPF*-overexpressing mutants revealed that 318 of the 440 upregulated genes in OE9 were repressed in WT plants, while 207 of the 475 downregulated genes in OE9 were induced in WT under drought stress (Lin et al. 2019). These results clearly highlight the putative role for trehalose in regulating the drought-responsive gene expression.

Consistently, similar results on trehalose accumulation in response to drought stress were observed in an experiment (Santana-Vieira et al. 2016) with two 2-year-old *Citrus* rootstocks, *Rangpur lime* ("RL", drought avoidance strategy adopter) and *Sunki maravilha* ("SM", drought-tolerance strategy adopter), ungrafted, grafted with their reciprocal graft combination or with shoot scions of two commercial citrus varieties: *Valencia orange* ("VO") and *Tahiti acid lime* ("TAL"). The eight different combinations obtained were subjected to drought stress by irrigation interruption until leaf water potential dropped to − 2 MPa (severe drought stress condition). After harvesting, plants were rehydrated for 48 h before the final harvesting. The experiment lasted 17 days during which multiple attributes were analysed. Among them, ABA, trehalose, and soluble sugar content had interesting expression profiles. In general, plants exhibited higher carbohydrate levels under severe drought stress in both leaves and roots as observed in the above-mentioned *Arabidopsis* studies. Interestingly, trehalose and sucrose contents increased under severe dehydration in roots of grafted or ungrafted SM rootstocks (drought tolerance adopter) and then decreased after 48 h of rehydration, while no significant changes were observed in plants with RL rootstocks. Soluble sugars act as osmoprotectant, but they also play a role against oxidative damage. Moreover, recent studies suggested that trehalose may have an antioxidant function as a direct ROS scavenger (Santana-Vieira et al. 2016). Higher carbohydrate content in fine roots could be explained by the fact that roots are the first organs to sense water deficit and consequently activate the necessary defence mechanisms to prevent drought-induced damages. Moreover, lowering in stomatal conductance was observed to be preceded by both

ABA and trehalose accumulation, suggesting for trehalose a role in facilitating ABA signalling to guard cells.

The discovery of trehalose pathway in plants is rather recent and much work is still to be done. However, even if to date there are not sufficient data on trehalose metabolism in woody plants, it can be hypothesized that the upregulation of specific trehalose biosynthetic genes could enhance plant tolerance to drought and other abiotic stresses.

ABA signalling

Abscisic acid (ABA) is a phytohormone involved in plant growth and in its response to different types of biotic and abiotic stresses. In the first stages of a plant life, ABA is essential for seed formation, dormancy and subsequent germination (Neves et al. 2013). Once the plant is well developed, ABA is still important in promoting growth as well as in triggering multiple defence mechanisms in response to various stresses, especially drought (Neves et al. 2013).

ABA is synthesized from carotenoids in a complex process that occurs largely in chloroplasts and ends in cytoplasm (Seo and Koshiba 2002):

(1) Zeaxanthin, which is formed from carotenoid in previous reactions, is converted into all-*trans*-violaxanthin by a two-step epoxidation catalysed by the enzyme zeaxanthin epoxidase (ZEP).
(2) All-*trans*-violaxanthin is converted in the xanthophylls 9-*cis*-neoxanthin and/or 9-*cis*-violaxanthin. The conversion from violaxanthin to neoxanthin is probably mediated by a neoxanthin synthase (Ikegami et al. 2009).
(3) 9-*cis*-neoxanthin and 9-*cis*-violaxanthin undergo an oxidative cleavage catalysed by the enzyme 9-*cis*-epoxycarotenoid dioxygenase (NCED), to obtain xanthoxin.
(4) Finally, xanthoxin is translocated from the plastids to the cytoplasm where it is converted in ABA. In *Arabidopsis*, xanthoxin is first converted to abscisic aldehyde by a dehydrogenase reductase and subsequently oxidized to ABA by an aldehyde oxidase (Ikegami et al. 2009).

Guard cells are specialized cells present in pairs and mainly located in leaf epidermis where they regulate gas exchanges. They are positioned to form a pore when they are turgid, named stomata, through which CO_2, O_2 and H_2O can pass in a process named transpiration (E). During drought stress, ABA accumulates in the leaves to induce stomatal closure by promoting the efflux of anions and K^+ ions from the guard cells which lose water and become flaccid, closing by this way the stomata. This pattern has been largely reported in many experiments with both herbaceous and woody plants. For instance, in Santana-Vieira's (2016) study, all citrus plant combinations showed increased ABA content in both leaves and roots in response to drought stress, while stomatal conductance (g_s), net photosynthetic rate (A) and transpiration (E) decreased in an inversely proportional way. *Sunki maravilha*, which adopts a drought-tolerance mechanism, accumulated significantly more ABA than *Rangpur lime* in their leaves and recovered more efficiently from severe drought. Similar results were obtained in an experiment with two olive trees, *Chemlali* (drought tolerant) and *Chetoui* (more sensitive to drought), subjected to water deficiency for 30 days (Guerfel et al. 2009). Both plants increased their ABA content and gradually reduced their g_s as the water became less available. Higher ABA levels in response to water deficiency were also observed in *Arabidopsis thaliana* (Ikegami et al. 2009). Therefore, it is possible to confirm that ABA production is triggered by drought stress and that this is a ubiquitous mechanism adopted by a wide range of plant species. Gomes et al. (2004) demonstrated the link between leaf water potential (Ψ_{leaf}), stomatal conductance (g_s), transpiration rate (E), CO_2 assimilation (A), CO_2 intercellular concentration (C_i) and ABA content in 30-month-old *Pêra* orange tree grafted on *Rangpur lime* and exposed to 10 days of drought stress, followed by 10 days of recovery. On the 7th day, when Ψ_{leaf} drastically lowered and drought stress became severe, g_s, E and A decreased, while ABA and C_i increased. ABA, which is de novo synthesized (Ikegami et al. 2009), gradually started to be produced soon after the onset of drought when Ψ_{leaf} at 2:00 p.m. (hereafter named Ψ_2) reached − 1.0 MPa, but its highest accumulation was drastically induced during severe drought stress at Ψ_2 values around − 3.5 MPa. Concurrently, total stomatal closure only occurred at $-3.0\ MPa < \Psi_2 < -3.5\ MPa$. Figure 6 (from Gomes et al. 2004) clearly shows that ABA and g_s are closely related in an inversely proportional relationship. Thus, as the leaf water potential decreases due to prolonged drought, ABA de novo biosynthesis begins to promote stomatal closure.

Decreases in g_s are accompanied with a decline in photosynthetic rate, but facilitate water retention and allow the plant to survive longer. However, it is still unknown whether under drought condition ABA is first synthesized in the roots or in the shoots, as many studies had contrasting results. While some evidences suggest that ABA is produced in the roots, the first organs to sense the lack of water, and then translocated to the leaves through xylem sap flow (Neves et al. 2013; Gomes et al. 2004; Guerfel et al. 2009), others state the opposite with the leaves being the first to synthesize ABA and subsequently promote its transport to the roots. Ikegami et al. (2009) showed that in *Arabidopsis*, when the leaves were exposed to drought, but the roots were kept in well-watered conditions, ABA accumulation occurred only in leaves; in contrast, when drought stress was applied

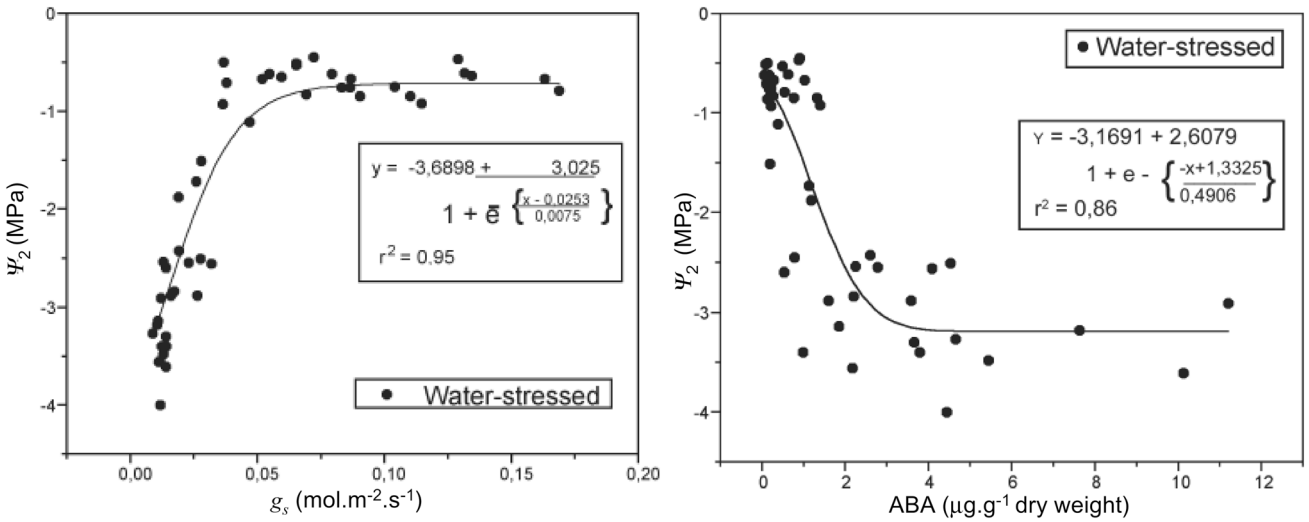

Fig. 6 Leaf water potential at 2:00 p.m. (Ψ_2) versus stomatal conductance (g_s) (left panel) and abscisic acid concentration (right panel) of "Pêra" orange trees submitted to drought stress. Data obtained from 50 observations (from Gomes et al. 2004)

to the roots, only a slight increase in ABA content was detected. Hence, this study suggests that ABA biosynthesis in response to drought stress first occurs in leaves.

NCED is the enzyme that catalyses the formation of xanthoxin, an ABA precursor. It belongs to the wider NCEDs family, which comprises multiple members that promote ABA biosynthesis during different stages of plant life cycle. In many studies, NCED was reported to be highly expressed during water deficiency conditions (Neves et al. 2013); in particular, NCED3 was found to be the enzyme responsible for ABA production in *Arabidopsis thaliana* during drought stress (Pedrosa et al. 2017). This led to the characterization of NCEDs from other species and, more recently, to the creation of transgenic plants overexpressing the genes coding for these enzymes to improve drought tolerance. Transgenic approaches that increased ABA production often resulted in plants with a better resistance to water scarce environments. When *CsNCED3*, the homolog from *Rangpur lime*, was introduced in *Nicotiana tabacum* under the control of the constitutive promoter *CaMV35S* (Pedrosa et al. 2017), transgenic plants subjected to 10 days of drought stress exhibited enhanced drought tolerance by closing the stomata much earlier than WT tobacco. Interestingly, even though transgenic tobacco had lower values of g_s throughout the experiment, its photosynthetic rate (A) remained similar to that of the WT. However, not all transgenic lines exhibited higher ABA levels compared to WT; this may indicate that increased ABA production might trigger its catabolism. Therefore, *CsNCED3* overexpression led to an improved drought tolerance by promoting stomatal closure to withhold as much water as possible, without negatively affecting the photosynthetic rate. In addition, transgenic tobacco showed reduced H_2O_2 content compared to the WT suggesting that NCED and ABA accumulation may promote the expression of ROS-scavenging enzymes.

In this context, overexpressing genes involved in ABA biosynthesis cannot be ruled out to realize more resistant plants to drought stress.

Proline accumulation

Proline is an amino acid involved in plant stress responsiveness. Although it is suggested that it has an important role in embryo development and in floral transition (Kaur and Asthir 2015), its accumulation mainly occurs under abiotic stress condition. In this context, it is thought that proline is an osmoprotectant involved in osmotic adjustment, in the stabilization of proteins and other subcellular structures, in ROS scavenging, in heavy metal chelation, in the activation of genes expressing stress-protective proteins/molecules and in the regulation of intercellular osmolarity to reduce the efflux of water. Moreover, it stabilizes the redox balance and its accumulation in chloroplasts helps maintain a correct $NADP^+$ pool (Kaur and Asthir 2015; Fang et al. 2015). Proline is synthesized by two different pathways that take place in different organelles (Fig. 7):

(a) Glutamate pathway takes place in chloroplast and consists in two successive reduction reactions: the first one is catalysed by pyrroline-5-carboxylate synthetase (P5CS), which is the rate limiting enzyme (and thus the most important in proline biosynthesis during stresses), while the second reaction is catalysed by the P5C reductase (P5CR).

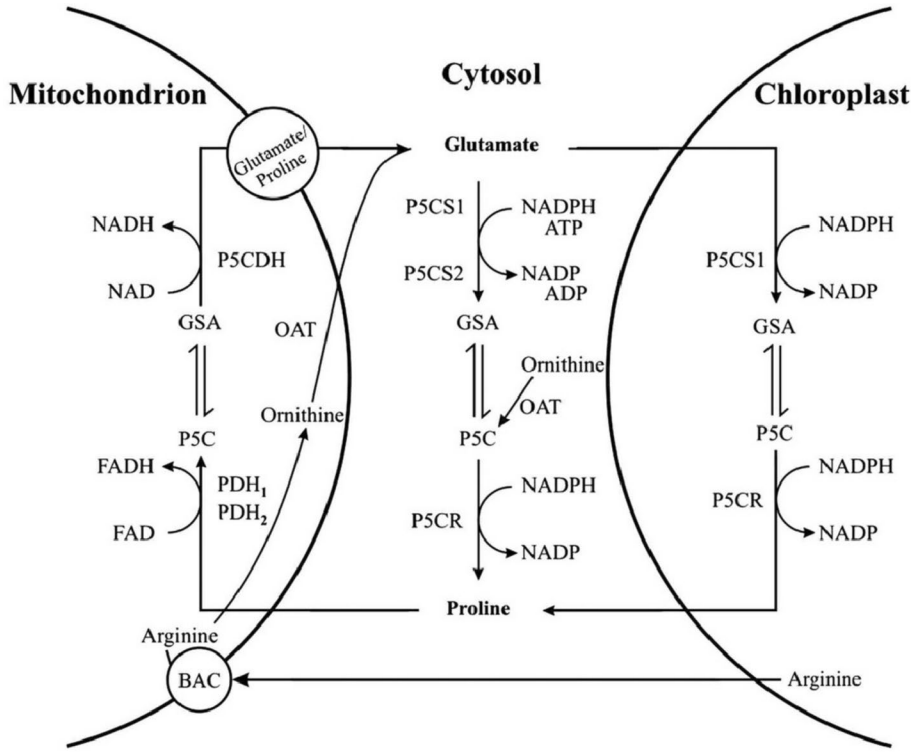

Fig. 7 Schematic representation of proline metabolism in different cell organelles. *P5CS* pyrroline-5-carboxylate synthetase, *P5CR* δ-pyrroline-5-carboxylate reductase, *PDH* proline dehydrogenase, *P5CDH* pyrroline-5-carboxylate dehydrogenase, *BAC* basic amino acid transporter, *GSA* glutamate-γ-semialdehyde, *OAT* ornithine aminotransferase (from Kaur and Asthir 2015)

(b) Ornithine pathway occurs in mitochondria and consists in the transamination of ornithine into P5C, catalysed by ornithine-δ-aminotransferase (OAT), which is then converted into proline.

Interestingly, proline content decreased in *Arabidopsis* knockout mutant for the *P5CS* gene, while no variations were observed in the *OAT*-defective mutant (Kaur and Asthir 2015). This result suggests that the glutamate pathway is the main route for proline biosynthesis, focusing the transgenic approaches on the genes involved. When the stress ends, proline content is restored to its initial level through the catabolic pathway which takes place in mitochondria with the help of the enzymes proline dehydrogenase (PDH) and P5C dehydrogenase (P5CDH) (Fig. 7). The reduction of NAD^+ to NADH during proline catabolism gives a consistent amount of reducing power to the cell that can be used to synthesize ATP. In fact, the oxidation of just one proline molecule can produce up to 30 ATP units (Kaur and Asthir 2015). Thus, proline catabolism provides energy that may facilitate plant recovery from stress (Fig. 8).

It has been frequently observed that proline content increases during drought stress. Higher proline levels were detected in the roots and the leaves of two citrus cultivars (*Carrizo citrange* and *Cleopatra mandarin*) when exposed both to single drought stress and to the combination of drought stress + heat stress (Zandalinas et al. 2017). Similar results were observed by Hussain et al. (2018) in six different citrus rootstocks subjected to drought. Interestingly, in both studies proline content was found to be higher in more sensitive plants compared to tolerant ones. During oxidative stress, proline acts as an ROS scavenger by binding to hydrogen peroxide and to the hydroxyl radical, creating stable adducts with them (Kaur and Asthir 2015); this reduces the lipid peroxidation and contributes to alleviating the oxidative damage. Therefore, the higher accumulation of proline in more sensitive genotypes compared to more tolerant ones may be explained by the lower antioxidant defence mechanism, suggesting for proline the role of counterbalancing the lack of antioxidant activity (Hussain et al. 2018).

Proline role in plants is still being investigated, and transgenic approaches designate proline as an interesting target for genetic engineering to enhance drought resistance in woody crops. In fact, de Carvalho et al. (2013) showed that a citrus cultivar (*Citrus paradisi* × *Poncirus trifoliata*) overexpressing the *Vigna aconitifolia P5CS* gene exhibited increased proline content, higher antioxidant enzyme activity and lower MDA level when exposed to 20 days of drought stress. Compared to WT plants, transgenic lines did not show leaf rolling after the drought treatment and exhibited a lower expression of some antioxidants even before the onset of stress. This study demonstrates that there is a positive correlation between proline accumulation and the regulation of antioxidant gene expression.

Transgenic approaches confirmed the link between increased proline content and a general upregulation of the

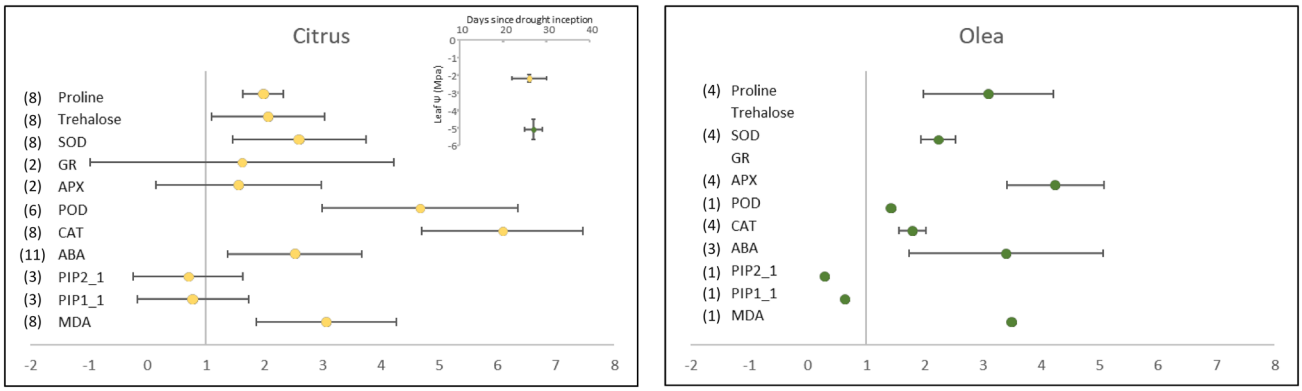

Fig. 8 Effect sizes of the drought stress (mean ± 95% CI, CI is confidence interval) on several molecular traits involved in the response. The sample size for each group is given on the left y-axis. The treatment effect (drought) is statistically significant if the 95% CI of the effect size does not overlap with the line. The insert illustrates the mean leaf Ψ versus the mean duration of the drought experiment ± 95% CI for the selected studies

antioxidant defence mechanism, which resulted in a better resistance to water deficiency conditions. Hence, genetic engineering aimed at increasing the enzyme production involved in proline biosynthesis deserves to be pursued.

Compilation of data and database analysis

A small database was compiled of available published papers providing quantitative data on the five selected drought resistance strategies in the two selected woody crops *Citrus* sp. and *Olea europea*. For this constraint, the database totalized only 32 entries, 22 for *Citrus* and 10 for *Olea* (Table 1). Both species were reported as cultivars and in terms of different rootstocks and shoot scions combinations. Very few papers reported quantitative data for transgenic cultivars, so they were excluded from data analysis. In particular for AQPs, some papers were excluded as no relative expression patterns were reported, but only pictures of blottings. All the selected compounds were measured on leaf except the AQPs on olive roots. For each molecular variable, all available data were scaled to the same unit: activity for enzymes, concentration for ABA, trehalose, proline and MDA, relative gene expression for AQPs. For each species record, information was added regarding the leaf water potential and the duration of drought stress treatment.

The approach adopted for data analysis was that of the response ratio, i.e. the ratio of the measured variable in treatment to control groups, which is commonly used as a measure of the proportionate change that results from an experimental manipulation. A true meta-analysis method examining both within-experiment and between-experiment variations was not applicable because of the very small database; therefore, only between-experiment variations are considered.

Data show that under drought stress condition, all the antioxidant activities, as well as proline, trehalose and ABA concentrations, increased, and the opposite was shown for the aquaporins. ABA concentration and AQPs gene expression showed a similar pattern for both species, although AQPs values were close to the null effect. Antioxidants and proline responses were different. Proline concentration resulted being higher in olive than citrus; the antioxidant profiles differed particularly for APX, POD and CAT activities, which was lower for APX and higher for POD and CAT in citrus than in olive, respectively. Despite that the range of drought duration was similar for the selected experiments and lasted on average 26 days, leaf water potential was twofold lower in olive than in citrus cultivars. Unfortunately, the cultivar feature was not analysed because of the small number of records, but the respective variance falls within the confidence interval shown in the graphs.

Discussion

Plants respond in a complex way to drought stress by inducing and/or reducing the expression of hundreds of genes that, to date, are still being studied. In the present work, five main strategies adopted by plants in response to drought stress (PIPs expression, antioxidant activity, ABA signalling, trehalose and proline accumulation) have been identified and investigated, and their genetic engineering has been designated as a potential solution to improve drought resistance in plants.

Data investigation highlighted a leaf water potential significantly lower in olive than citrus cultivars within a comparable range of drought duration. To this lower mean value, similar increases corresponded for most of the investigated traits except for APX, POD and CAT activities,

Table 1 Details of studies describing experiment duration, measurement interval, evaluated molecular parameters and main results in *Olea* and *Citrus* cultivars

	Name of the crop	Experiment duration	Measurement interval	Evaluated parameters	Results
AQPs					
Wei et al. 2019	*Sanhuhongju* (Citrus) *Sanhuhuahong* (Citrus)	2 weeks of drought stress	7th and 14th day of drought treatment	Drought-responsive expression of CsAQPs	Most CsPIP and sTIP genes were differentially downregulated in the roots of two citrus cultivars under drought condition
Secchi et al. 2007	*Olea europea* (2-year-old)	4 weeks of drought stress + 4 weeks of rewatering	3rd, 4th, 6th and 8th week of the experiment + first 3 days of rewatering	Drought-responsive expression of *OePIP2.1* aquaporin gene	In drought-stressed plants the level of *OePIP2.1* transcript abruptly dropped and started increasing after 3 days of rewatering
Secchi et al. 2014	*Populus tremula* × *Populus alba* (Populus)	1 to 5 days of drought stress + 1 day of rewatering	Every day between 9 am and 4 pm	Xylem embolism in a *PIP1* downregulated *Poplar* tree	The downregulation of PIP1 genes negatively affects the embolism refilling rate and increases embolism formation
Wang et al. 2019	Transgenic *Arabidopsis thaliana* (4-week-old) overexpressing *ScPIP1*	20 days of drought stress + rewatering period	At the end of the experiment	[MDA]; [PRO] …in a transgenic *Arabidopsis* plant overexpressing *ScPIP1 Jojoba* aquaporin gene	The overexpression of *ScPIP1* gene enhances drought and osmotic tolerance by increasing PRO content and decreasing MDA content
Rodríguez-Gamir et al. 2011	*Cleopatra mandarin*, *Poncirus trifoliata*, *hybrid 030,115* (Citrus) 15-months-old	70 days of water stress	On day 43 of the experiment	Drought-responsive expression of PIP aquaporin genes in roots	The downregulation of PIP genes in *Cleopatra mandarin* and in the *hybrid* during drought stress resulted in a better drought tolerance
Perez-Martin et al. 2014	*Olea europaea* (5-year-old)	13 days of drought stress + 6 weeks of recovery	Every day	Drought-responsive expression of *OePIP2.1* and *OePIP1.1* aquaporin genes	A general downregulation is observed during drought stress. However, *OePIP1.1* transcript increased at the onset of the stress. During the recovery period a general upregulation can be observed
Araújo et al. 2019	*Olea europaea* (7-month-old)	1 month of drought stress + 10 days of recovery	At the end of the drought period and after the recovery	Drought-responsive expression of *OePIP2.1* and *OePIP1.1* aquaporin genes	PIPs seem to be more active during olive recovery. Particularly, OePIP2.1 was, in general, more abundant in plants recovering from drought stress condition

Table 1 (continued)

	Name of the crop	Experiment duration	Measurement interval	Evaluated parameters	Results
Antioxidants					
Hussain et al. 2018	*Volkameriana lemon, Brazilian sour orange, Carrizo citrange, Eureka lemon, Gada dahi, Rangpur lime* (Citrus)	24 days of drought stress	Leaf attributes were measured on the 6th, 12th, 18th and 24th day Root attributes were measured at the end of the experiment	[MDA]; [H_2O_2]; [Pro]; Antioxidant enzymes activities: [SOD]; [CAT]; [POD]	Drought stress causes oxidative stress, which increases ROS production that negatively affects metabolic activities. A general upregulation of antioxidant enzymes is observed during the stress
Zandalinas et al. 2017	*Carrizo citrange, Cleopatra mandarin* (Citrus) 1-year-old	7 days of heat stress + 1 day of drought stress	At the end of the experiment	[MDA]; [H_2O_2]; [Pro]; Antioxidant enzymes activities: [SOD]; [CAT]; [APX]	Increased ROS production negatively affects metabolic activities Better antioxidant enzyme activities and proline accumulation confer enhanced drought and heat tolerance
Sofo et al. 2005	*Coratina* (Citrus) 2-year-old	20 days of drought stress	Leaf attributes were measured after 0, 4, 8, 12, 16 and 20 days Root attributes were measured after 0, 8, 16 and 20 days	Antioxidant enzyme activities: [SOD]; [CAT]; [POD]; [APX]	Antioxidant enzymes have a higher activity when the plant is in moderate and severe stress condition
Trehalose					
Santana-Vieira et al. 2016	*Rangpur lime*, *Sunki maravilha, Valencia orange*, *Tahiti acid lime* (Citrus) 2-year-old	17 days of both drought and recovery period	At the end of the stress period and 48 h after rewatering	[Soluble sugars]; [ABA]	Following drought stress, trehalose accumulation was found to be higher in more drought-tolerant plants
Iordachescu and Imai 2008	*Arabidopsis thaliana*	24 h (in silico analysis)	0.25, 0.5, 1, 3, 6, 12 and 24 h from the beginning of the experiment	Drought-responsive expression of TPSs and TPPs (genes involved in trehalose biosynthesis)	Trehalose differently accumulates in response to various abiotic stresses
Lin et al. 2019	Transgenic *Arabidopsis thaliana* (1-week-old) overexpressing *AtTPPF* gene	- First experiment: 26 days of drought stress + 2 days of rewatering - Second experiment: 24 h of drought stress	At the beginning and at the end of each experiment	Survival rate of *Arabidopsis* plants overexpressing *AtTPPF* gene (1stexperiment) [H_2O_2]; [sucrose] … in *Arabidopsis* seedlings overexpressing *AtTPPF* gene (2ndexperiment)	*AtTPPF*-overexpressing lines exhibited enhanced drought tolerance and increased soluble sugars level (mainly sucrose), while loss-of-function mutants were more sensitive to drought

Table 1 (continued)

	Name of the crop	Experiment duration	Measurement interval	Evaluated parameters	Results
ABA					
Gomes et al. 2004	*Pera x Rnagpur lime* (Citrus) 30-months-old	10 days of drought stress + 10 days of rewatering	Every day	[ABA]; stomatal conductance (Gs); leaf water potential (Ψ_{leaf})	During drought stress, Ψ_{leaf} decreased while ABA content increased promoting stomatal closure and reducing Gs
Ikegami et al. 2009	*Arabidopsis thaliana*	4 h of drought stress	0, 1, 2.5 and 4 h from the onset of drought	[ABA] in roots; [ABA] in leaves; Gs	Under drought stress ABA content increased and its biosynthesis occurred in the leaves
Guerfel et al. 2009	*Chemlali* and *Chetoui* (*Olea europaea*) 3-month-old	30 days of drought stress	Every 6 days between 9:00 and 10:00 am	[ABA]; Gs; Ψ_{leaf}	Under drought stress ABA content increased in both plants
Pedrosa et al. 2017	Transgenic *Nicotiana tabacum* overexpressing *CsNCED3*	10 days of drought stress	On alternate days between 9 and 11am	[ABA]; Gs; photosynthetic rate (A)	Transgenic plants exhibited enhanced drought tolerance and A
Neves et al. 2013	*Rnagpur lime, Sunki maravilha* (Citrus)	40 days of drought stress	At 4 different intervals of soil moisture level ($m^3\ m^{-3}$): 0,29 – 0,28 (control) 0,20 – 0,19 0,17 – 0,16 0,15 – 0,14	[ABA]; Ψ_{leaf}	In response to WS, [ABA] levels in plants increased to reduce water loss by closing the stomata
Kitsaki and Drossopoulos 2005	*Koroneiki* (Olea europaea)	30 days of drought stress	At the end of the WS period	[ABA]; Ψ_{leaf}	ABA and Ψ_{leaf} are negatively correlated
Proline					
de Carvalho et al. 2013	Transgenic *Swingle citrumelo* (Citrus) overexpressing *VaP5CS* gene 4-year-old	20 days of water stress	Measurements were taken when Ψ_{leaf} reached: -1.3 MPa (well watered); -2.5 MPa (moderate stress); -3.9 (severe stress); -1.8 MPa (rehydration)	[Pro]; Antioxidant enzyme activities: [SOD]; [CAT]; [APX]; [GR]	*P5CS* overexpression resulted in enhanced proline accumulation, higher antioxidant expression and better resistance to drought stress
Ben Ahmed et al. 2009	*Chemlali, Meski, Picholine* (Olea europaea)	150 days of drought stress	At the end of the treatment	[proline]; Antioxidant enzyme activities: [SOD]; [CAT]; [APX]	The ability of olive trees to upregulate the enzymatic antioxidant system and to accumulate proline could be effective in water-limited environments
Sofo et al. 2004	*Coratina* (Olea europaea)	20 days of drought stress	0, 4, 8, 12, 16 and 20 days from the beginning of the experiment (between 6 and 7 am)	[Proline]; [MDA]	The accumulation of proline indicates its possible role in drought tolerance, while the increased MDA content shows that oxidative damages are a consequence of WS

which resulted in the former being lower and the other two higher in citrus than in olive, respectively, and a slightly higher proline concentration for olive. Consequently, it may be assumed that *Citrus* cultivars prefer to adopt higher leaf water potential maintenance and antioxidant activities via CAT and POD pathways strategies against severe drought stress, whereas *Olea* cultivars seem to adopt stronger osmoprotectant strategy (higher [proline]) and antioxidant activities via the ascorbate–glutathione pathway. AQPs and ABA mechanisms seem to be involved to the same extent, although citrus maintains a higher leaf water potential. The lack of significant AQPs gene expression was not particularly surprising, as they are referred to the leaves. Investigations at root level might highlight a different response.

The significant difference between the leaf water potential may be ascribed to different strategies of drought resistance at leaf cell structure scale. Lo Gullo and Salleo (1988) clearly showed such a different strategy between *Olea oleaster* and other two sclerophyll species, *Ceratonia siliqua* and *Laurus nobilis*. Despite an equal degree of sclerophylly (in terms of the ratio of leaf dry weight to surface area), *Olea* is more drought tolerant than *Ceratonia* and *Laurus*, as it shows a drastic and prolonged diurnal drop in leaf Ψ coupled with a higher rate of water loss, whereas *Ceratonia* and *Laurus* show an avoidance strategy achieved by water spending or water saving and rapid recovering from minimal water losses through a drastic lowering of leaf water potential. The drastic lowering is achieved by the higher modulus elasticity (rigidity) of the parenchyma cell wall in *Laurus* leaf (Lo Gullo and Salleo 1988). The leaf Ψ values observed in this study for *Citrus* sp. leaves are similar to those reported for *Ceratonia* or *Laurus*, suggesting the adoption of a drought avoidance mechanism at leaf anatomical scale. Furthermore, the higher proline concentration in olive leaves may further explain their ability to further lower the water potential, reinforcing the involvement of proline accumulation in drought tolerance mechanisms. Unfortunately, it is necessary for *Olea* to fill the gap of information on trehalose concentration which shares the osmoprotective activity with proline. Therefore, since the contribution of structural leaf trait like the modulus elasticity of parenchyma cell walls play a key role in the resulting leaf water potential (Lo Gullo and Salleo 1988), transgenic optimization for *Olea* cultivars should address mainly stiffening of leaf parenchyma cell walls, helping by this way the recovery from minimal water loss. For *Citrus* cultivars, enhancement of the constitutive metabolic dehydration tolerance by molecular traits such as osmoprotectant and/or antioxidants activities seems to be more functional.

Investigated data are leaf scaled, but relationships with the hydraulic system cannot be ruled out, as genetically induced growth limitation is reported to enhance the expression of root and shoot water channels belonging to the PIP1 and PIP2 subfamilies in two-year-old olive saplings, suggesting a possible compensation of reduced plant hydraulic conductance because of lower root mass, and a contribution to alleviate limitation to whole-plant growth (Lovisolo et al. 2007).

The other interesting outcome from this study has been the higher APX activity in olive compared to citrus. This non-enzymatic antioxidant pathway has been proved to be more important than other antioxidant pathways under stronger single stress pressure or in combined stress conditions (heat plus drought) (Koussevitzky et al. 2008). Indeed, olive leaves experience a higher water deficit, and the significant increase of APX activity could be considered among the main mechanisms developed by olive trees for the protection of chloroplasts, which under stress conditions present sustained electron flows and are the main producers and targets of ROS action (Ben Ahmed et al. 2009).

It is worth highlighting that the age for 27 out of 32 entries ranged from 1 to 2 years, with three cases of 7 months and one case each of 5- and 40-year-old olive trees. Thus, caution should be exercised when extrapolating from the responses of seedlings to environmental conditions to the responses of older trees. Nevertheless, outcomes from seedlings' or saplings' responses are important for the management of the early establishment performance of these crops under field conditions.

Conclusions

From a strict genetic improvement point of view, overexpressing all the above-mentioned genes simultaneously does not seem feasible and, moreover, it could have negative effects on plant growth. However, some of the investigated mechanisms have improved plant resistance against more than just one abiotic stress. In fact, as oxidative damages occur with almost every abiotic stress, enhancing antioxidant activity could result in a better tolerance to drought as well as to other adverse conditions such as cold or high salinity. Similarly, trehalose and proline are involved in protecting plant from drought, salt, osmotic and oxidative stresses, thanks to their osmoprotectant and switch in roles for the expression of hundreds of stress-responsive genes. These multiple alterations to metabolism may result in a higher crop yield as, during their life cycle, agronomic plants face a combination of different abiotic stresses under field conditions such as drought, cold, heat stresses and other unfavourable environmental conditions. In contrast, leaf AQPs and ABA are mainly involved in regulating transpiration and in preventing water losses; thus, their transgenic approaches would help create drought-tolerant crops, but would not improve their resistance against other abiotic stresses.

In conclusion, integration of the molecular approaches with morpho-physiological analyses that closely examine the structure–function relationship for the organs mainly involved in the drought response are necessary if progress is to be made in developing effective approaches for manipulating and improving drought resistance.

References

Afzal Z, Howton T, Sun Y, Mukhtar M (2016) The roles of Aquaporins in plant stress responses. J Dev Biol 4:9. https://doi.org/10.3390/jdb4010009

Araújo M, Ferreira de Oliveira JMP, Santos C et al (2019) Responses of olive plants exposed to different irrigation treatments in combination with heat shock: physiological and molecular mechanisms during exposure and recovery. Planta 249:1583–1598. https://doi.org/10.1007/s00425-019-03109-2

Ben Ahmed C, Ben Rouina B, Sensoy S et al (2009) Changes in gas exchange, proline accumulation and antioxidative enzyme activities in three olive cultivars under contrasting water availability regimes. Environ Exp Bot 67:345–352. https://doi.org/10.1016/j.envexpbot.2009.07.006

Caverzan A, Casassola A, Patussi Brammer S (2016) Reactive oxygen species and antioxidant enzymes involved in plant tolerance to stress. In: Shanker AK, Shanker C (eds) Abiotic and biotic stress in plants—recent advances and future perspectives. InTech, pp 463–480

Cruz F, Julca I, Gómez-Garrido J et al (2016) Genome sequence of the olive tree. Olea Europaea Gigascience. https://doi.org/10.1186/s13742-016-0134-5

de Carvalho K, de Campos MKF, Domingues DS et al (2013) The accumulation of endogenous proline induces changes in gene expression of several antioxidant enzymes in leaves of transgenic *Swingle citrumelo*. Mol Biol Rep 40:3269–3279. https://doi.org/10.1007/s11033-012-2402-5

de Gomes M, M de A, Lagôa AMMA, Medina CL, et al (2004) Interactions between leaf water potential, stomatal conductance and abscisic acid content of orange trees submitted to drought stress. Brazilian J Plant Physiol 16:155–161. https://doi.org/10.1590/S1677-04202004000300005

Denaxa N-K, Damvakaris T, Roussos PA (2020) Antioxidant defense system in young olive plants against drought stress and mitigation of adverse effects through external application of alleviating products. Sci Hortic 259:108812. https://doi.org/10.1016/j.scienta.2019.108812

Fang Y, Xiong L (2015) General mechanisms of drought response and their application in drought resistance improvement in plants. Cell Mol Life Sci 72:673–689. https://doi.org/10.1007/s00018-014-1767-0

Guerfel M, Beis A, Zotos T et al (2009) Differences in abscisic acid concentration in roots and leaves of two young Olive (Olea europaea L.) cultivars in response to water deficit. Acta Physiol Plant 31:825–831. https://doi.org/10.1007/S11738-009-0298-Z

Huang H, Ullah F, Zhou D-X et al (2019) Mechanisms of ROS regulation of plant development and stress responses. Front Plant Sci. https://doi.org/10.3389/fpls.2019.00800

Hussain S, Khalid MF, Saqib M et al (2018) Drought tolerance in citrus rootstocks is associated with better antioxidant defense mechanism. Acta Physiol Plant 40:135. https://doi.org/10.1007/s11738-018-2710-z

Ikegami K, Okamoto M, Seo M, Koshiba T (2009) Activation of abscisic acid biosynthesis in the leaves of Arabidopsis thaliana in response to water deficit. J Plant Res 122:235–243. https://doi.org/10.1007/s10265-008-0201-9

Iordachescu M, Imai R (2008) Trehalose biosynthesis in response to abiotic stresses. J Integr Plant Biol 50:1223–1229. https://doi.org/10.1111/j.1744-7909.2008.00736.x

Kaur G, Asthir B (2015) Proline: a key player in plant abiotic stress tolerance. Biol Plant 59:609–619. https://doi.org/10.1007/s10535-015-0549-3

Kitsaki CK, Drossopoulos JB (2005) Environmental effect on ABA concentration and water potential in olive leaves (Olea europaea L. cv "Koroneiki") under non-irrigated field conditions. Environ Exp Bot 54:77–89. https://doi.org/10.1016/j.envexpbot.2004.06.002

Koussevitzky S, Suzuki N, Huntington S et al (2008) Ascorbate Peroxidase 1 plays a key role in the response of *Arabidopsis thaliana* to stress combination. J Biol Chem 283:34197–34203. https://doi.org/10.1074/jbc.M806337200

Latowski D, Surówka E, Strzałka K (2010) Regulatory role of components of ascorbate–glutathione pathway in plant stress tolerance. In: Anjum N, Chan MT, Umar S (eds) Ascorbate-glutathione pathway and stress tolerance in plants. Springer, Dordrecht. https://doi.org/10.1007/978-90-481-9404-9_1

Lawlor DW (2013) Genetic engineering to improve plant performance under drought: physiological evaluation of achievements, limitations, and possibilities. J Exp Bot 64:83–108. https://doi.org/10.1093/jxb/ers326

Li Q, Qi J, Qin X et al (2020) CitGVD: a comprehensive database of citrus genomic variations. Hortic Res 7:12. https://doi.org/10.1038/s41438-019-0234-3

Lin Q, Yang J, Wang Q et al (2019) Overexpression of the trehalose-6-phosphate phosphatase family gene AtTPPF improves the drought tolerance of Arabidopsis thaliana. BMC Plant Biol 19:1–15. https://doi.org/10.1186/s12870-019-1986-5

Lo Gullo MA, Salleo S (1988) Different strategies of drought resistance in three Mediterranean sclerophyllous trees growing in the same environmental conditions. New Phytol 108:267–276. https://doi.org/10.1111/j.1469-8137.1988.tb04162.x

Lovisolo C, Secchi F, Nardini A et al (2007) Expression of PIP1 and PIP2 aquaporins is enhanced in olive dwarf genotypes and is related to root and leaf hydraulic conductance. Physiol Plant 130:543–551. https://doi.org/10.1111/j.1399-3054.2007.00902.x

Neves DM, Filho MAC, Bellete BS et al (2013) Comparative study of putative 9-cis-epoxycarotenoid dioxygenase and abscisic acid accumulation in the responses of Sunki mandarin and Rangpur lime to water deficit. Mol Biol Rep 40:5339–5349. https://doi.org/10.1007/s11033-013-2634-z

Pedrosa AM, Cidade LC, Martins CPS et al (2017) Effect of overexpression of citrus 9-cis-epoxycarotenoid dioxygenase 3 (CsNCED3) on the physiological response to drought stress in

transgenic tobacco. Genet Mol Res 16:1–10. https://doi.org/10.4238/gmr16019292

Penna S (2003) Building stress tolerance through over-producing trehalose in transgenic plants. Trends Plant Sci 8:355–357. https://doi.org/10.1016/S1360-1385(03)00159-6

Perez-Martin A, Michelazzo C, Torres-Ruiz JM et al (2014) Regulation of photosynthesis and stomatal and mesophyll conductance under water stress and recovery in olive trees: correlation with gene expression of carbonic anhydrase and aquaporins. J Exp Bot 65:3143–3156. https://doi.org/10.1093/jxb/eru160

Rodríguez-Gamir J, Ancillo G, Aparicio F et al (2011) Water-deficit tolerance in citrus is mediated by the down regulation of PIP gene expression in the roots. Plant Soil 347:91–104. https://doi.org/10.1007/s11104-011-0826-7

Santana-Vieira DDS, Freschi L, Da Hora Almeida LA et al (2016) Survival strategies of citrus rootstocks subjected to drought. Sci Rep 6:1–12. https://doi.org/10.1038/srep38775

Secchi F, Zwieniecki MA (2014) Down-regulation of plasma intrinsic protein1 aquaporin in poplar trees is detrimental to recovery from embolism. Plant Physiol 164:1789–1799. https://doi.org/10.1104/pp.114.237511

Secchi F, Lovisolo C, Schubert A (2007) Expression of OePIP2.1 aquaporin gene and water relations of *Olea europaea* twigs during drought stress and recovery. Ann Appl Biol 150:163–167. https://doi.org/10.1111/j.1744-7348.2007.00118.x

Seo M, Koshiba T (2002) Complex regulation of ABA biosynthesis in plants. Trends Plant Sci 7:41–48. https://doi.org/10.1016/S1360-1385(01)02187-2

Sofo A, Dichio B, Xiloyannis C, Masia A (2004) Lipoxygenase activity and proline accumulation in leaves and roots of olive trees in response to drought stress. Physiol Plant 121:58–65. https://doi.org/10.1111/j.0031-9317.2004.00294.x

Sofo A, Dichio B, Xiloyannis C, Masia A (2005) Antioxidant defences in olive trees during drought stress: changes in activity of some antioxidant enzymes. Funct Plant Biol 32:45–53. https://doi.org/10.1071/FP04003

Wang X, Gao F, Bing J et al (2019) Overexpression of the Jojoba aquaporin gene, ScPIP1, enhances drought and salt tolerance in transgenic Arabidopsis. Int J Mol Sci 20:1–13. https://doi.org/10.3390/ijms20010153

Wei Q, Ma Q, Ma Z et al (2019) Genome-wide identification and characterization of sweet orange (Citrus sinensis) aquaporin genes and their expression in two citrus cultivars differing in drought tolerance. Tree Genet Genomes 15:1–13. https://doi.org/10.1007/s11295-019-1321-1

Zandalinas SI, Balfagón D, Arbona V, Gómez-Cadenas A (2017) Modulation of antioxidant defense system is associated with combined drought and heat stress tolerance in citrus. Front Plant Sci 8:1–10. https://doi.org/10.3389/fpls.2017.00953

Zargar SM, Nagar P, Deshmukh R et al (2017) Aquaporins as potential drought tolerance inducing proteins: towards instigating stress tolerance. J Proteomics 169:233–238. https://doi.org/10.1016/j.jprot.2017.04.010