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Original article

Tetraploid Carrizo citrange rootstock (*Citrus sinensis* Osb. × *Poncirus trifoliata* L. Raf.) enhances natural chilling stress tolerance of common clementine (*Citrus clementina* Hort. ex Tan)

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ABSTRACT

Low temperatures can disturb the development, growth and geographic distribution of plants, particularly cold-sensitive plants in the Mediterranean area, where temperatures can reach seasonally low levels. In citrus crops, scion/rootstock combinations are used to improve fruit production and quality, and increase tolerance to biotic and abiotic stresses. In the last decade, several studies have shown that tetraploid citrus seedlings or rootstocks are more tolerant to abiotic stress than their respective diploid. The objective of this study was to test whether the use of tetraploid rootstocks can improve the chilling tolerance of the scion. We compared physiological and biochemical responses to low seasonal temperatures of common Clementine (*Citrus sinensis* Osb. × *Poncirus trifoliata* L. Raf.) grafted on diploid and tetraploid Carrizo citrange rootstocks, named C/2xCC and C/4xCC, respectively. During the coldest months, C/4xCC showed a smaller decrease in net photosynthesis (*P*n), stomatal conductance (G_s), chlorophyll fluorescence (F_v/F_m), and starch levels, and lower levels of malondialdehyde and electrolyte leakage than C/2xCC. Specific activities of catalase (CAT), ascorbate peroxidase (APX) and dehydroascorbate reductase (DHAR) were higher in C/4xCC during the cold period, whereas chlorophyll, proline, ascorbate and hydrogen peroxide (H_2O_2) levels and superoxide dismutase (SOD) activity did not vary significantly between C/4xCC and C/2xCC throughout the study period. Taken together, these results demonstrate that tetraploid Carrizo citrange rootstock improves the chilling tolerance of common clementine (scion) thanks to a part of the antioxidant system.

1. Introduction

Polyploidy is a common biological phenomenon and a major force of plant evolution (Chen, 2007; Soltis and Soltis, 2009). In nature, 50–70% of angiosperms have undergone at least one episode of polyploidization (Masterson, 1994). In citrus, tetraploid can be divided into two categories − doubled diploid or allotetraploid (Stebbins, 1947) − resulting from either somatic chromosome doubling or sexual reproduction via 2n gametes, respectively. Doubled diploid citrus genotypes arise from somatic chromosome doubling and from intraspecific hybridization or self-fertilization through 2n gametes, with the subgenomes in doubled diploid thus being considered identical (Aleza et al., 2011). Allotetraploids citrus inherited subgenomes from two different parents after interspecific hybridization. Tetraploidization may lead to specific genome expression changes that can in turn induce phenotypic changes that trigger an increase in productivity and

Abbreviations: ABA, abscisic acid; Asa, reduced ascorbate; APX, ascorbate peroxidase; CAT, catalase; CBFs, C-repeat-binding factor; CO_2, carbon dioxide concentration; DHA, oxidized ascorbate; DHAR, dehydroascorbate reductase; F_0, minimal value of chlorophyll a fluorescence; F_m, maximum fluorescence; F_v, variable fluorescence of photosystem II; F_v/F_m, the maximal quantum yield of photosystem II; FE%, electrolyte leakage percentage; G_s, stomatal conductance; H_2O_2, hydrogen peroxide; QA, quinone acceptor; MDA, malondialdehyde; $O_2^{\cdot -}$, superoxide radical; OH·, hydroxyl radical; P_{net}, net photosynthesis rate; PPFD, photosynthetic photon flux density; PSII, photosystem II; ROS, reactive oxygen species; SOD, superoxide dismutase; tAsa, total ascorbate; TBA, thiobarbituric acid; TCA, trichloroacetic acid; C/2xCC, clementine grafted onto diploid carrizo citrange rootstock; C/4xCC, clementine grafted onto tetraploid Carrizo citrange rootstock

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efficiency, especially by enhancing stress tolerance (Tan et al., 2015). Polyploid species are more common in extreme environments, with low temperatures, high radiation, low fertility soils and dry conditions (Brochmann et al., 2004; Ehrenforfer, 1980; Levin, 1983). Based on several reports, the use of tetraploid genotypes is an alternative way to improve stress tolerance in many crops (Saleh et al., 2008; Meng et al., 2012; Tan et al., 2015; Ruiz et al., 2016a,b,c). In a previous study, we showed that tetraploid Rangpur lime (*Citrus limonia* Osbeck) rootstock improved the water deficit tolerance of Valencia scion compared to the use of diploid Rangpur lime rootstock grafted with Valencia scion (Allario et al., 2013).

Rootstock/scion combinations are frequently employed in the citrus industry to improve fruit production and quality, and the tolerance to biotic and abiotic stresses. The rootstocks are selected for root traits linked to resistance to pests and pathogens from the soil but also to various abiotic stresses such as salinity, drought, floods and cold. Roots subjected to cold have decreased water absorption due to increased root hydraulic resistance (Kramer, 1940) and decreased membrane permeability (McElhaney et al., 1973). This results in decreased nutrient and water absorption causing a disruption of plant growth and development (Ahn et al., 1999). Usually, citrus rootstocks are diploid with a basic chromosome number x = 9 (Krug, 1943). Citrus rootstocks are propagated by polyembryonic seeds since citrus species are partially apomictic. Spontaneous doubled diploid (4×) seedlings may arise from chromosome set doubling of nucellar cells (maternal tissue) (Cameron and Frost, 1968).

Carrizo citrange (*Citrus sinensis Osb. x Poncirus trifoliata L. Raf.*) and its parent *Poncirus trifoliata* are two rootstocks classically used in Corsica. When grown as seedlings, these genotypes have deciduous leaves and undergo winter dormancy. In *Poncirus trifoliata,* this marked difference was probably acquired during the evolutionary process in northeastern Asia when the trees were exposed to cold temperatures (Ziegler and Wolfe, 2017). However, when grafted, these cultivars are no longer deciduous and their cold tolerance is much more limited, although the use of *Poncirus trifoliata* and Carrizo citrange rootstock is still one of the most effective ways to boost cold tolerance. However, little is known regarding the mechanisms that allow the rootstock to improve the scion's cold tolerance.

When plants are exposed to cold temperatures, profound changes in gene expression are observed. The C-repeat-binding factor (CBFs) pathway is known to play a very important role in the response to cold stress (Chan et al., 2016). Huang et al. (2015) recently showed that, under cold conditions, ICE1 (inducer of CBF expression 1) of *Poncirus trifoliata* modulates polyamine levels through interactions with arginine decarboxylase. This leads to lower hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\cdot -}$) contents, and higher activity of antioxidant enzymes, such as superoxide dismutase and catalase. Thus, it is likely that Carrizo citrange inherited this capacity to better tolerate cold from its *Poncirus trifoliata* parent. Carrizo citrange rootstock is also widely used since it is tolerant to *Citrus Tristeza Virus* and flooding. In addition, it promotes good fruit productivity and quality (Pérez-Clemente et al., 2012).

In the Mediterranean region, the climate is dry and hot during the summer and relatively cold during the winter. Regions such as Corsica, which is the most northern citrus growing area, can sometimes have relatively low temperatures. For example, during the coldest months (January and February) of winter 2010–2011, the average daily minimum air temperatures were 4.1 and 4.6 °C (Santini et al., 2013) in Corsica. Below 13 °C, the development and growth of citrus is disturbed (Ribeiro and Machado, 2007).

Low temperatures can interfere with photosynthesis and redox homeostasis in cells (Asada, 1999). This disruption of the photosynthetic machinery induces an over-production of reactive oxygen species (ROS) leading to an oxidative stress, one of the most harmful consequences of cold stress (Asada, 2006). The main ROS are $O_2^{\cdot -}$, H_2O_2 or the hydroxyl radical (OH·). They are produced on a regular basis in biological pathways as by-products or signal transducers; however, an excess of ROS can cause oxidative damage to organic molecules like deoxyribonucleic acid (DNA) mutation, protein denaturation and membrane lipid peroxidation (Mittler, 2002; Apel and Hirt, 2004). The latter can lead to changes in the permeability and fluidity of the membrane phospholipid bilayer and can significantly alter cellular integrity. Plants possess antioxidant enzymes (superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and dehydroascorbate reductase (DHAR)) and non-enzymatic antioxidants (ascorbate and proline) for scavenging ROS. This antioxidant system has already been shown to have a crucial role in the cold tolerance of plants (Sabehat et al., 1998).

In this study, we tested whether the use of tetraploid rootstocks can increase the chilling stress tolerance of the citrus graft. The effects of seasonal temperature fluctuations were investigated on common clementine (*Citrus clementina Hort. ex Tan*) grafted on diploid and tetraploid Carrizo citrange rootstocks.

2. Materials and methods

2.1. Plant material and growth conditions

The experiment was performed on 40-year-old Clementine trees (*Citrus clementina* Hort. ex Tan; SRA 63; ICVN 0100059) grafted onto Carrizo citrange rootstock (*Citrus sinensis* (L.) Obs. x *Poncirus trifoliata* (L.) Raf.; ICVN 0110476). Bark samples were collected (1 cm^2 area cut from below the bud union) from each single rootstock to determine its ploidy using flow cytometry (Froelicher et al., 2007). In the orchard, 11 tetraploid Carrizo citrange were identified. Four clementine trees grafted onto diploid Carrizo citrange (*Citrus sinensis (L.) Obs.* x *Poncirus trifoliata (L.) Raf*) (ICVN 0110476) rootstock (C/2xCC) and four clementine trees grafted onto the respective tetraploid form of the same rootstock (C/4xCC) were selected for investigation. All trees had the same South orientation and were a similar height above ground (about 1.5 m). Trees were grown under organic farming conditions in an orchard located in Moriani, Corsica, France (42° 23′ 08″ N and 09° 31′ 47″E). Measurements and sampling were carried out from October 2015 to May 2016. The same study was also carried out in 2014–2015 and the results obtained were identical (data not shown). The coldest months were January, February and March. We focused on the coldest sunny days of those three months, where the minimum temperature was −1, 0.8 and 0.9 °C, respectively (Table 1). Climatic data were collected throughout the sampling period (Table 1).

Resistance to chilling stress was evaluated through measurement of the main photosynthetic traits (net photosynthesis, stomatal conductance, chlorophyll content and chlorophyll *a* fluorescence), electrolyte leakage, starch levels, oxidative status (malondialdehyde, hydrogen peroxide); the contribution of the antioxidant system was assessed by enzymatic (scavenging and recycling enzymes) and non-enzymatic assays (ascorbate, proline). For each physiological measurement, 3 mature leaves per tree and per genotype were used (12 replicates). Leaves were selected on 1-year branches subjected to the same light exposure (East, Southeast). Each parameter was measured between 9 am and 11 am. For biochemical analyses, 4 samples of 20 fully expanded leaves were sampled for each genotype (4 replicates) once a month from October 2015 to May 2016. This sampling was carried out as described in previous studies (Allario et al., 2013; Hussain et al., 2012; Santini et al., 2012) in which four replicates were used for biochemical parameters. Harvested leaves were immediately immersed in liquid nitrogen and then stored at −80 °C. Before performing biochemical studies, each leaf sample was ground to a fine powder in liquid nitrogen.

2.2. Measurements of gas exchange and chlorophyll content

Leaf net photosynthetic rate (P_{net}) and stomatal conductance (G_s)