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Polyploidization alters constitutive content of volatile organic compounds (VOC) and improves membrane stability under water deficit in Volkamer lemon (*Citrus limonia* Osb.) leaves

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ABSTRACT

In *Citrus* species chromosome doubling naturally occurs in somatic embryos and doubled diploid plants often show better adaptation to adverse environmental condition. To understand the molecular determinants of stress acclimation, we examined the response to water deficit in diploid (2×VL) and doubled diploid (4×VL) seedlings of Volkamer lemon (*Citrus limonia* Osb.) assessing the profile of constitutive volatile organic compound (VOC) in control and stressed conditions. Physiological parameters and leaf volatile compound profiles were measured during water deficit and 24 h after rehydration of plants to field capacity. Net photosynthesis and stomatal conductance were reduced in water stressed leaves, with no significant differences between 2×VL and 4×VL plants. Malondialdehyde concentration, a marker of lipid peroxidation of cellular membranes, was significantly more higher in stressed 2×VL leaves than in 4×VL. The blend of constitutive VOC was different in control leaves being oxygenated monoterpenoids more abundant in 2×VL leaves, and monoterpenoids more abundant in 4×VL leaves. Water deficit did not stimulate biosynthesis of terpenoids, whereas accumulation of trans-2 hexenal, a green leaf volatile (GLV) synthesized after membrane denaturation, was observed in stressed leaves of 2×VL leaves, but not in 4×VL leaves. Semiquantitative PCR showed an increase of the expression of *HPL*, the gene encoding for hydroperoxidase lyase which catalyzes 2-hexenal formation, only in 2×VL plants. The expression of the putative dehydration transcription factor *DREB2A* was also observed only in 2×VL water stressed plants. This work shows that level of ploidy may alter constitutive content of GLV by Citrus, therefore likely affecting plants capacity of protection and interaction with other organisms. Whereas diploid and double diploid plants showed similar physiological responses to water deficit, a biochemical marker indicated that membranes of double diploid leaves were more resistant to the stress. These results provide intriguing insights into the regulation of terpenoids and oxylipins pathways as a function of polyploidization in a non-model plant species.

1. Introduction

Citrus is one of the most important fruit crop in the world and its production is challenged by many environmental constraints. Citrus varieties are routinely grown on rootstocks to help them coping with a range of stressful conditions (Levy and Syvertsen, 2004; Tadeo et al., 2008). Citrus rootstocks are propagated through polyembryonic seeds and the seedlings regenerated from nucellar embryos are genetically identical to the maternal plant. The majority of cultivated citrus genotypes has a partially apomictic reproduction with a somatic embryogenesis of nucellar cells induced by the gamete fertilization (Aleza et al., 2011). Doubled

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diploid seedlings in apomictic genotypes are considered to arise from somatic chromosome doubling of nucellar embryo cells and should be genetically identical, at the qualitative level, to the seed source tree (Rao et al., 2008). In reality, the phenotype of doubled diploids is modified in various traits, such as a reduced growth vigor and enlargement of cells and organs (Comai, 2005). Polyploidy has been an important determinant in plant evolution, facilitating plant invasiveness and the capability to successfully colonize habitats characterized by strong fluctuating environmental conditions (Beest et al., 2012). Furthermore, recent studies also demonstrate that genome doubling confers to plants also a better adaptability to various environmental stresses included a higher tolerance to salinity (Mouhaya et al., 2010; Jiang et al., 2013) and water deficit (Li et al., 2009). In citrus plants, Allario et al. (2013) found that in control conditions 4× rootstocks constitutively overexpressed a set of genes putatively involved in water deficit tolerance. Podda et al. (2013) found higher levels of antioxidant enzymes, such as superoxide dismutase and ascorbate peroxidase, in 4× Cleopatra and Willow leaf mandarin than the respective 2× plants challenged with salt stress.

Drought stress is a major environmental factor that affects plant growth and development and are expected to increase with climate change (Dai, 2010; Centritto et al., 2011).

Plants release into the surrounding atmosphere a vast range of volatile organic compounds (VOC) which have a role as infochemicals in biotic interaction (Blée, 2002; Niinemets et al., 2013) and also in abiotic stress acclimation. VOCs are often considered as a mechanism for plants to respond to stresses and alleviating their negative consequences (Loreto and Schnitzler, 2010).

The terpenoids, and the green leaf volatiles (GLVs), including 2-hexenal (*cis*,*trans* aldehydes C6), are two families of volatile organic compounds (VOC) abundantly emitted by stressed plants (Feussner and Wasternack, 2002; Dudareva et al., 2004). Green leaf volatiles are well-studied as compounds produced by plants in response to wounding (Loreto et al., 2006; Brilli et al., 2011), to pathogenic infection (Blée, 2002; Scala et al., 2013) and to herbivory (Maja et al., 2014). However, induction of GLV after abiotic stress was also observed. Emission of (E)-2-hexenal was detected in a photoinhibition sensitive Arabidopsis mutant after exposure to intense light conditions (Loreto et al., 2006), in tomato under heat and cold stresses (Copolovici et al., 2012), and in tobacco after exposure to ozone (Beauchamp et al., 2005), and under other photooxidative stress condition (Mano et al., 2010). Recently, it has been suggested that 2-hexenal can act as endogenous signal chemical inducing abiotic-responsive genes (Kramell et al., 2000; Savchenko et al., 2014; Yamauchi et al., 2015).

Profiling of VOC has been largely used to evaluate biotic and abiotic stress response in citrus plants, for example in response to Citrus Tristeza Virus (Cheung et al., 2015), winter flooding and salinity (Velikova et al., 2012) or to blue light (Pallozzi et al., 2013). However, to our knowledge, no study has examined the impact of ploidy combined with water deficit on VOC profile in citrus plants. Thus, the aim of this work was to compare the VOC content in 2× and 4× Volkamer lemon plants, in control and water stress conditions, to gain new insight on the role of VOC in water stress deficit and on the association of VOC profile with the genetic structure of citrus plants.

2. Material and methods

2.1. Plant material and growth conditions

Thirty diploid (2×VL) and thirty doubled diploid (4×VL), six-month-old seedlings of Volkamer lemon (*Citrus limonia* Osb.) were obtained from seeds of fruit picked in adult trees maintained in the citrus germplasm collection (INRA/CIRAD CRB Citrus), San Giuliano, Corsica (France). The ploidy status of 2× and 4× plants was previously checked and confirmed by flow cytometry (Partec I) according to Froelicher et al. (2007). The clonal propagation by nucellar embryogeneis was verified by genotyping the offspring using 13 SSR markers according to protocols and markers developped by Luro et al. (2008). Markers are listed in the Supplementary Table S1. Non conform Volkamer lemon plants were discarded. The selected plants were transplanted in 3 L pots containing commercial fresh soil, and then transferred in a chamber of the Institute for Sustainable Plant Protection in Florence, which was equipped to grow plants for 6 months under the following controlled condictions: photoperiod of 16 h, with day/night of 25–32 °C/18–20 °C, and relative humidity varying daily between 50 and 80%. The photon flux density at leaf level was 300–350 $\mu mol\ m^{-2}\ s^{-1}$,supplied mainly with cool lights. The plants were regularly irrigated during the week and were fertilizated with Bayfolan (Bayer) (NPK 5–7–8 + microelements), every 15 days.

2.2. Water deficit

Water deficit experimental design involved two phases (Supplementary Fig. S1). In the first phase, twenty-four plants for each genotype were irrigated at field capacity and the excess water was allowed to drain overnight. After draining, the pots were weighed to determine the weight at field capacity ($Initial_{potweight}$). Each pot was then enclosed in a plastic bag that was tied around the stem to prevent soil evaporation. Then, twelve plants for each genotype were water-stressed by withholding water, while other 12 plants for each genotype continued to be watered to field capacity (control plants). Water deficit development was followed and parameterized measuring the pot weight and calculating the fraction of transpirable soil water (FTSW) (Sinclair and Ludlow, 1986; Brilli et al., 2013). Every two days, during the water deficit experiment, all plastic bags were unwrapped to weigh plants ($nDay_{potweight}$) and to water control plants compensating water loss by transpiration. The physiologically lower limit of available soil water was defined as the FTSW at which stomatal conductance approached zero (Sinclair and Ludlow 1986; Brilli et al., 2013). Once this level was achieved, the water-stressed plants were weighed to determine the final pot weight ($Final_{potweight}$). Then, the FTSW was calculated for each single pot as: $FTSW = (nDay_{potweight - Finalpotweight})/(Initial_{potweight} - Final_{potweight})$ and water was provided to all plants to reach the initial pot weight. Leaves for metabolomic and biochemical analysis were harvested at (100% of FTSW) (T_0), 50% of FTSW (T_1), 0% of FTSW (T_2), and after 24 h from irrigation of water-stressed plants (R).

2.3. Leaf net photosynthesis and stomatal conductance

During water deficit experiment, net photosynthesis and stomatal conductance were recorded with a portable photosynthesis system IRGA equipped with an integrated fluorometer (LI-6400, Li-Cor Inc., Nebraska, USA (model 6400; Li-Cor, Lincoln, NE)). Measurements were performed on the central portion of the first fully expanded leaf using a photosynthetic photon flux density of 600 $\mu mol\ m^{-2}\ s^{-1}$, a leaf temperature of 25 °C, a relative humidity near 40% and a CO_2 concentration of 390 $\mu mol\ mol^{-1}$. Measurements were repeated on 3–5 plants for each genotypes, at the same times when destructive sampling was carried out.

2.4. Proline extraction and determination

Proline was extracted according to Bates et al. (1973). Briefly, 20 mg of leaf were crushed in liquid N_2 with mortar and pestle and homogenized in 70:30 ethanol:water at 95 °C for 20 min. The