Research article

Silencing of ascorbate oxidase results in reduced growth, altered ascorbic acid levels and ripening pattern in melon fruit

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ABSTRACT

Ascorbate oxidase (AO, EC 1.10.3.3) is a copper-containing enzyme localized at the apoplast, where it catalyzes the oxidation of ascorbic acid (AA) to dehydroascorbic acid (DHA) via monodehydroascorbic acid (MDHA) intermediate. Despite it has been extensively studied, no biological roles have been definitively ascribed. To understand the role of AO in plant metabolism, fruit growth and physiology, we suppressed AO expression in melon (*Cucumis melo* L.) fruit. Reduction of AO activity increased AA content in melon fruit, which is the result of repression of AA oxidation and simultaneous induction of certain biosynthetic and recycling genes. As a consequence, ascorbate redox state was altered in the apoplast. Interestingly, transgenic melon fruit displayed increased ethylene production rate coincided with elevated levels of 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (ACO, EC 1.14.17.4) activity and gene expression, which might contribute to earlier ripening. Moreover, AO suppressed transgenic melon fruit exhibited a dramatic arrest in fruit growth, due to a simultaneous decrease in fruit cell size and in plasmalemma (PM) ATPase activity. All the above, support for the first time, the *in vivo* AO participation in the rapid fruit growth of *Cucurbitaceae* and further suggest an alternative route for AA increase in ripening fruit.

Abbreviations: AAT, alcohol acyl-transferase; ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC oxidase; APX, ascorbate peroxidase; ATP, adenosine triphosphate; AWF, apoplastic content; DAP, days after hand pollination; DHAR, dehydroascorbic acid reductase; Exp, expansin; GalDH, L-galactono-1,4-lactone dehydrogenase; GGP/VTC2, GDP-L-galactose phosphorylase; GME, GDP-D-mannose 3' 5'-epimerase; GPP/VTC4, L-galactose-phosphate phosphatase; MDHAR, monodehydroascorbic acid reductase; MPG1, polygalacturonase; NPT, neomycin phosphotransferase; PM, plasmalemma; WT, wild-type.

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1. Introduction

Ascorbic acid (AA), vitamin C, is a chemical compound required for human diet (Fenech et al., 2018; Mellidou et al., 2017; Mellidou et al., 2019; Smirnoff, 2018). AA is found in all cell compartments where acts as an antioxidant and scavenger against the so-called reactive oxygen species (ROS), which are natural by-products of aerobic cell metabolism and photosynthesis (Mellidou and Kanellis, 2017; Venkatesh and Park, 2014). Apoplastic AA that accounts for 10% of total AA content, acts as a first defense line against hostile environments. Apoplastic AA levels represent a sensitive mechanism by which abiotic and biotic stresses can be monitored and consequently activate plant defense-responses (Bellini and De Tullio, 2019; Foyer and Noctor, 2005). Changes in the levels and redox status of apoplastic AA in response to environmental stresses have been reported (Sanmartin et al., 2003). Moreover, several AA-deficient mutants and transgenic plants of *Arabidopsis thaliana* and *Nicotiana tabacum* showed increased sensitivity to ozone and to other oxidative stresses (Conklin et al., 1996; Fotopoulos et al., 2006; Sanmartin et al., 2003; Veljovic-Jovanovic et al., 2001) demonstrating the role of AA in plant defense (Macknight et al., 2017). Nonetheless, in the case of *Arabidopsis vtc* mutants, the different sensitivity to ozone cannot be solely justified by differences in AA levels, indicating the complexity of AA physiology and metabolism in plants (Conklin et al., 2000). In addition, as a unique enzyme cofactor, AA plays a crucial role in essential plant biosynthetic pathways such as the xanthophyll cycle, the ethylene biosynthesis and the synthesis of cell wall hydroxyproline rich proteins (HRGP) (Bulley and Laing, 2016; Smirnoff and Wheeler, 2000), pointing to AA involvement in plant development as well (Mellidou et al., 2017; Plumb et al., 2018). Indeed, *Arabidopsis vtc* mutants showed reduced cell growth as well as abnormalities in cell shape (Fenech et al., 2018; Mellidou and Kanellis, 2017).

There is also evidence supporting AA participation in cell division and/or cell expansion (Smirnoff, 2000, 2018). AA accumulates in actively growing meristems and in young tissues (De Gara and Tommasi, 1999), whereas root quiescent centers and dormant buds have little or no AA (Arrigoni et al., 1992; Kerk and Feldman, 1995). Early reports showed that cells normally competent to divide are held in the G1 phase of the cell cycle if AA content is lowered (Liso et al., 1984). Conversely, AA supplement to root cells resulted in a faster cell proliferation (Arrigoni et al., 1997; Kerk and Feldman, 1995) indicating a close correlation between AA levels and root meristem mitotic activity. The low AA levels in root quiescent centers and dormant buds were attributed to the presence of ascorbate oxidase (AO), an enzyme extensively studied with yet no ascribed biological roles. Regulation of AO gene expression by growth promoters and suppressors (Pignocchi et al., 2003a; Sanmartin et al., 2007) pointed to a possible AO role in controlling cell expansion (Kato and Esaka, 1999, 2000; Smirnoff, 1996). Although the mechanism is not completely understood, oxidation of apoplastic AA by AO produces monodehydroascorbate (MDHA), and regeneration of MDHA back to AA could result in hyperpolarization of the plasma membrane and acidification of the apoplast, with the subsequent activation of the ion transport that leads to vacuole and cell enlargement (Gonzalez-Reyes et al., 1994).

Ascorbate oxidase is a unique plant enzyme found in high amounts in cucurbits (De Tullio et al., 2013). It was first described in cabbage (Svent-Györgi, 1931) and, since then numerous reports have dealt with its purification and characterization (Diallinas et al., 1997; Esaka et al., 1992; Lin and Varner, 1991; Moser and Kanellis, 1994). Fast growing plant tissues showed the highest AO activity independently of the organ nature, fruit or leaf. Melon AO activity and expression reach high levels during fruit development, just before the climacteric rise (Diallinas et al., 1997; Sanmartin et al., 2007), while cucumber AO mRNA, protein, and activity are mainly found in fruit epidermis (Yoshida et al., 1994). In this context, AO might play a significant role in controlling cell wall metabolism during fruit ripening and participate in the fast softening process observed in melon fruit, by the loss of hypodermal mesocarp

membrane integrity (Lester and Stein, 1993).

Although the main biosynthetic pathway of AA in plants has been elucidated (Bulley and Laing, 2016; Mellidou et al., 2017; Smirnoff, 2018; Wheeler et al., 1998), regulation of AA biosynthesis or pool size in plants is poorly understood. Increasing the AA pool by exogenous AA application strongly inhibits its endogenous synthesis in embryonic axes of germinating pea seedlings (Pallanca and Smirnoff, 2000). Therefore, it seems that plants might control AA biosynthesis according to its endogenous pool size through a feedback mechanism (Bulley and Laing, 2016; Fenech et al., 2018; Pallanca and Smirnoff, 2000; Smirnoff, 2018). Due to its nutritional importance, a better understanding of AA biosynthesis and its metabolism will help in improving AA content of dietary plants. Engineering plants with increased AA level will not only improve the nutritional value but it will also favor plants' tolerance to abiotic and biotic stresses. Since the AA content in a cell is a result not only of its biosynthesis, but also of its catabolism and turnover, work is also needed to establish the role of the latter enzymes in controlling AA levels and redox state. To define this, we transformed melon plants with sense and antisense *Cucumis melo* AO4 (*CmA04*) gene constructs to study the effects of altered AO levels in melon fruit. Our results indicate that AO contributes to the rapid growth of the cucurbits by indirectly acting on PM H⁺-ATPase activity through the hyperpolarization of plasma-membrane and points out to an alternative route for controlling AA content during fruit development and ripening.

2. Materials and methods

2.1. Plant material and cloning procedures

Melon cv. Hale's Best Jumbo (Hollar Seed, Colorado, USA) and cv. Vedrantaiz Tezier (kindly provided by Prof. JC Pech, ENSAT Toulouse, France) seeds were used. Putative transgenic plants were transferred to sterile soil mix as soon as roots were well-developed and acclimated to greenhouse conditions. Flowers were hand-pollinated and tagged for date of fruit set and ripening state. For developmental and ripening studies, melon plants were grown in a greenhouse at 25 °C day/night at the premises of the School of Agricultural Sciences, Aristotle University of Thessaloniki. Fruit were picked at various developmental stages [25-, 30-, 32-, 34-, 36-, 38-, and 40-days after hand pollination (DAP)], and the inner and outer areas of fruit mesocarp were separated using a scalpel blade, prior to snap-freezing in liquid nitrogen and stored at −80 °C.

CmA04 full-length cDNA (Sanmartin et al., 2007) in the sense orientation was transferred into the pGA643 plant transformation vector (An et al., 1988) as an *Xba*I fragment, and in the antisense orientation at the *Hind*III-*Hpa*I sites, downstream of the constitutive *CaMV* 35S promoter. Constructs were sequenced and introduced into two *Agrobacterium tumefaciens* strains, EHA105 and C58, by the freeze-thaw method (Holsters et al., 1978).

2.2. Plant transformation

Melon transformation was performed following previously described protocol (Fang and Grumet, 1990) with minor modifications. Seeds were deoated, sterilized in 15% (v/v) commercial bleach (5.25% sodium hypochlorite) with a drop of Tween-20 for 10 min and then rinsed three times with sterile distilled water. Seeds were placed on hormone free Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with 0.8% agar and allowed to germinate. The culture growth room conditions were 25 °C with a 16 h-light/8 h-dark photoperiod provided by cool white fluorescent lamps (ca. 2500 lux). Four-day old cotyledons were cut in 4–6 pieces and incubated for 10 min in a diluted overnight *Agrobacterium* culture (1:10 in LB). Explants were blotted on filter paper, transferred abaxially to regeneration media [MR, MS supplemented with 0.88 mg/L indole acetic acid (IAA), 1.22 mg/L benzylaminopurine (BAP) and 0.26 mg/L abscisic acid and 3% sucrose] without

antibiotics and kept in co-culture for 3 days. Explants were washed three times with dH₂O, blotted dry and transferred onto MR with 175 or 200 mg/L kanamycin and 400 mg/L timentin. Regenerated green shoots were separated and placed on filter paper on a layer of liquid elongation media (MS supplemented with 0.1 mg/L BAP, 3% sucrose) with antibiotics and kept for 2–3 weeks. Elongated green shoots were transferred to rooting media (hormone-free MS with 3% sucrose, 400 mg/L timentin). As roots started developing, leaf samples (20–30 mg) were evaluated for the presence of neomycin phosphotransferase (NPT II) protein by ELISA assay (5 Prime-3 Prime, Inc, Boulder, CO) performed according to manufacturer's instructions. Regenerated T0 plants were transferred to sterile soil mix and sealed in a plastic bag to acclimate to greenhouse conditions. To provide sufficient T0 transgenic fruit for evaluation, plants were vegetatively propagated using a commercial rooting powder [0.06% (w/w) indole-3-butyric acid] and hand-pollinated to produce T1 progeny. For further characterization, CoAO4 transgenic plants were used.

2.3. Genomic DNA isolation and PCR screening

Genomic DNA was isolated from 1 to 2 g leaf tissue using the protocol described previously (Diallinas et al., 1997). For PCR screening, the presence of the sense insert was verified using 35S primer (5' ATCTTCGCAAGACCTTCC 3') and PAOII primer (5' GCAAATGGGGTTCAATATGGC 3'). For transgenic plants harboring the antisense construct, genomic DNA was amplified using the 35S and the AO4-UTR primers (5' TCTAGACATCTCCACAACTTCCCA 3'). PCR products were analyzed by Southern blot analysis as described earlier (Sambrook et al., 1989) to confirm that the amplified fragments corresponded to the expected transgene using as probe the *Cla*I-*Sph*I fragment of *CmA*O4 cDNA.

2.4. Southern and RNA blot analysis

Southern blot analysis of genomic DNA was performed to determine the T-DNA copy number. Genomic DNA (10 µg) was digested with *Hind*III, a restriction enzyme that cut only once in the T-DNA but not in the transgene. Digested genomic DNA was fractionated in a 0.8% agarose TAE gel run overnight at 50 V. The gel was transferred to a nylon membrane (Nytran® 0.45 Schleicher & Schuell) and hybridized at 65 °C with a *NPTII* specific probe.

Total RNA was extracted from melon stems and fruit as described before (Diallinas et al., 1997; Sanmartin et al., 2003). Total RNA (15 µg) was used on denaturing agarose/formaldehyde gels, transferred onto nylon membranes (Nytran® 0.45) and hybridized with specific radio-labeled probes (Supplementary Table 1) as applied previously (Sanmartin et al., 2003).

2.5. Protein extraction and enzyme activity assay

For total protein extraction, melon tissues (1 g) were ground in liquid nitrogen and extracted with 2 vol of extraction buffer containing 50 mM potassium phosphate pH 7.4, 1 M sodium chloride, 1 mM PMSF, 1.5% (w/v) PVPP and 10% glycerol. The mixture was homogenized with a Polytron and allowed to stand on ice for 20 min with occasional vortexing every 5 min. After centrifugation at 15,000g for 20 min at 4 °C, the supernatant was recovered and assayed for enzyme activity. Protein extraction of the soluble and particulate fractions was performed as previously described (Sanmartin et al., 2003). Soluble and particulate fractions were assayed directly for AO activity (Diallinas et al., 1997). One unit of AO activity was defined as the oxidation of 1 µmol AA min⁻¹ at 25 °C considering an extinction coefficient for ascorbic acid of 9.249 mM⁻¹ cm⁻¹ at 265 nm. All enzyme assays were carried out at 25 °C.

Plasma membranes were isolated in an aqueous two-phase system as described (Kjellbom and Larsson, 1984) with some modifications. In brief, melon fruit tissue was homogenized using Polytron PT10/35

homogenizer, in a medium containing 0.5 M sucrose, 5 mM ascorbic acid, 1 mM PMSF and 10 mM HEPES-KOH pH 7.5. Homogenate was filtered through four layers of cheesecloth and centrifuged for 10 min at 10,000 g. Supernatant was re-centrifuged for 60 min at 87,000 g (Beckman L7-55, SW28 rotor). Resulting pellets were suspended in a phase buffer (5 mM potassium phosphate pH 7.8, 3 mM KCl, 0.33 M sucrose) and separated in a 6.5% (w/w) two-phase system (6.5% PEG 3350, 6.5% Dextran T400, 0.33 M sucrose, 3 mM KCl, 5 mM potassium phosphate pH 7.8). The final upper phase was diluted 10-fold in washing buffer (0.25 M sucrose in HEPES-KOH, pH 7.5) and pelleted at 104,000 g for 120 min. Final plasma membrane pellets were suspended in membrane resuspension medium (0.25 M sucrose, 10% glycerol, 10 mM HEPES-KOH, pH 7.5) and stored at -70 °C until use. PM H⁺-ATPase activity in plasma membrane preparations was assayed by spectrophotometric determination of Pi released from ATP hydrolysis as previously described (Terry and Williams, 2002).

2.6. Analysis of ascorbic acid content

Total ascorbate was determined following extraction of 500 mg fruit tissue in 2 ml of ice-cold 6% (w/v) metaphosphoric acid containing 0.2 mM DTPA. Extracts were centrifuged at 10,000 g for 4 min at 4 °C and the supernatant recovered. For apoplastic content (AWF), thin fruit layers (500 mg) were vacuum infiltrated with 66 mM potassium phosphate (pH 3.0), 0.2 mM DTPA and 1 mM sodium azide. AWF was recovered into 100 µl of cold 6% (w/v) metaphosphoric acid and 0.2 mM DTPA after centrifugation for 10 min at 5,000 g at 4 °C, and then tissues were re-weighed in order to determine the volume of recovered AWF.

Ascorbic acid/dehydroascorbate (AA/DHA) content was determined using the spectrophotometric method described by Takahama and Oniki (1992), based upon the difference in absorbance at 265 nm before, and 3 min after the addition of AO (1 U/µl) to a 50 µl aliquot of extract in 60 mM potassium phosphate buffer (pH 6.3). Dehydroascorbate content was determined by measuring absorbance before and 5 min after adding 0.5 M DTT to a separate 50 µl aliquot of extract.

Cytosolic contamination of apoplastic fluid was monitored by assaying glucose-6-phosphate dehydrogenase (G6PDH) activity as marker (Lyons et al., 1999). The percentage activity with respect to the corresponding soluble symplastic fraction ranged between 0.1 and 2, which indicates a low contamination with intracellular components.

2.7. Ethylene production rate

Fruit were placed in sealed 20 L glass jars for 30 min and air samples were taken for ethylene determination. Ethylene concentration was determined by injecting the gas sample into a Varian 3300 gas chromatograph (Varian Instruments, Walnut Creek, CA) equipped with a flame ionization detector and a stainless column.

2.8. Firmness and weight determination

Whole fruit flesh firmness was measured using a Chatillon penetrometer fitted with a 7.9 mm tip, on two opposite sides after skin removal. Determination of the fruit weight was performed using an electronic scale.

2.9. Confocal laser scanning microscopy

In order to visualize cell walls, propidium iodide staining was used as a 10 µg/ml solution in water and tissues were incubated for 5 min (Truernit and Haseloff, 2008). Fluorescence was visualized using a Nikon C1 confocal laser-scanning microscope (CLSM; Nikon D-Eclipse C1; Nikon Inc., Melville, NY, USA) at 536 nm excitation and 617 nm emission. Images represent a specific dimension area of melon fruit tissues. Cell number was counted and averaged from 3 cross sections in each one of 3 independent biological replications. Cell size per cross

Fig. 1. Characterization of *CmA04* transgenic melon lines. **A**, Total RNA (~15 μg) isolated from WT and T0 transgenic young stems was analyzed by RNA blot with *CmA04* specific probes. Ribosomal RNA staining (rRNA) was used as loading control. **B**, Total AO specific activity was measured in young stems from WT, AsAO4 and CoAO4 transgenic melon plants. Results represent means from at least three different stems. Vertical bars showed standard deviations. Statistically significant differences are indicated with different letters above the bars. **C**, Genomic DNA (~10 μg) isolated from melon plants was digested with *HindIII*, fractionated in 0,8% agarose-TAE gels and hybridized with a *NPTII* specific probe. Ha, Hales; Ve, Vedrantais; S1, S2, plants transformed with AO4-S construct; G1, H1, E7 and F7, plants transformed with AO4-AS construct.

section was calculated using the circle area formula (πr^2), where r was calculated as the mean between cell maximum and minimum diameter divided by 2. Mean cell size was calculated for each one of the cells of three cross sections of three independent biological replications.

2.10. Statistical analyses

Data were analyzed by one way-analysis of variance (ANOVA, Duncan's multiple range test; except for Fig. 1C where Tukey test was applied; $P < 0.05$) using the SPSS software.

3. Results

3.1. Generation and evaluation of *CmA04* transgenic lines

Melon plants exhibit low transformation efficiency. Moreover, efficiency is affected by different factors such as genotype, environmental conditions, *Agrobacterium* strain and regeneration protocol used (Guis et al., 1998). In our experiments, we used cotyledons as starting plant material, and we determined the regeneration efficiency using different protocols (Supplementary Table 2). Cotyledons from two melon cultivars, Vedrantais and Hales, were transformed with two *Agrobacterium tumefaciens* strains, C58 and EHA105 both carrying *CmA04* in sense and antisense orientation. Although *CmA01* is mainly expressed in melon fruit, *CmA04* gene, abundant in vegetative tissues, melon ovaries and green immature fruit (Sanmartin et al., 2007) was chosen to possibly target both vegetative and fruit AO expression. Hales explants inoculated with *Agrobacterium* EHA105 strain showed identical regeneration frequencies at either 175 or 200 mg/L kanamycin, whereas explants inoculated with C58 strain showed a 2-fold decrease at the higher kanamycin concentration. Shooting frequency was similar on both antibiotic concentrations regardless of the *Agrobacterium* strain used (Supplementary Table 3). Although both melon genotypes could be transformed with *Agrobacterium* strain C58, Vedrantais exhibited higher transformation efficiency. In contrast, only Hales transgenic plants could be generated with EHA105 strain. Nine T0 transgenic melon lines, positive for the *NPTII* Elisa test, were identified out of 13.081 explants used (Supplementary Table 3), however F1 line did not develop further. PCR analysis combined with Southern blotting confirmed the presence of AO4 sense and antisense inserts (Supplementary Fig. 1). Two transgenic plants, originally named S1 and S2, carried the AO4 sense construct while the other seven (B1, E7, F7, H1, G1 and G2) were obtained using the AO-antisense (pAO4-AS) construct. B1 plants showed an aberrant growth and never developed completely, while G2 did not acclimate to greenhouse conditions.

To monitor whether the integration site of the AO sense or antisense constructs, into the melon genome, can influence *CmA04* transcript accumulation, we performed northern blot using total RNA from young stems (Fig. 1A). *CmA04* transcripts were hardly detected in stems of the G1, H1, E7 and F7 T0 antisense plants (hereafter referred to as AS-AO4 plants), while S1 and S2 T0 transgenic sense lines were almost depleted of *CmA04* transcripts, probably due to co-suppression of the endogenous gene (hereafter referred to as CoAO4 lines; Fig. 1B). Additionally, AO specific activity was determined in T0 melon transgenic young stems. AO activity paralleled *CmA04* transcript levels (Fig. 1A and B). Total AO specific activity showed a 2-fold decrease in T0 Hales AS-AO4 plants (G1 and H1), whereas T0 CoAO4 lines (S1 and S2) showed a 95% reduction in AO specific activity in stems (Fig. 1B). In addition, wild type Vedrantais stems showed higher AO values than Hales counterparts. Moreover, AO activity in Vedrantais AS-AO4 stems (E7 and F7) showed lower levels than wild type (Fig. 1B) but the differences were not statistically significant.

Southern analysis was applied to determine the number of T-DNA insertions in the transgenic lines using the *NPTII* gene as probe. All transgenic plants carried the *NPTII* gene, whereas wild type plants did not contain this gene (Fig. 1C). In addition, different copy numbers were

Fig. 2. Altering AO activity provokes an increased in AA content. **A**, Total AO specific activity was measured in melon ovaries (ov) and fruit at different developmental stages (25-, 32-, 34-, 36-, 38-, 40-days after pollination) of WT (white columns) and *CoAO4* transgenic melon plants (black columns). Results represent means from at least three individual fruit. **B**, AO specific activity was measured in the apoplast of fruit mesocarp at different mature ripe stages (32- and 36-days after pollination) of WT (white columns) and *CoAO4* transgenic melon plants (black columns). **C**, AA content in fruit apoplast. **D**, DHA content in fruit apoplast. **E**, AA redox state in fruit apoplast. In all graphs, vertical bars showed standard deviations. Statistically significant differences are indicated with different letters above the bars.

detected in the different transgenic plants. Transgenic T0 *CoAO4* plants S1 and S2 have a single copy of the T-DNA, while transgenic antisense plants showed multiple copies (Fig. 1C). T0 *AS-AO4* Hales plants harbored five to six copies whereas transgenic antisense Vedralta plants presented at least three copies of the T-DNA insertion (Fig. 1C). The similar pattern obtained within each pair of transgenic lines indicated that they could be derived from three different transformation events and that each line was a replicate of each other (S1 to S2, G1 to H1 and E7 to F7). Taken together, three independent transgenic lines were generated either by antisense or by co-suppressed silencing that showed reduced *CmAO4* transcript accumulation and AO enzymatic activity. Since *CoAO4-S2* contained a single copy of the transgene and showed the highest reduction in AO activity, we generated *CoAO4-S2* homozygous plants for further characterization.

3.2. Reduced AO levels increase AA content in melon fruit

To determine whether alteration of *CmAO4* expression levels affected enzyme activity, we measured total AO activity in different fruit developmental stages (Fig. 2A). AO activity was particularly high in wild

type ovaries in line with previous published data (Diallinas et al., 1997), whereas transgenic *CoAO4* plants displayed a 97% reduction compared to wild type plants. During fruit development, *CoAO4* plants showed a substantial reduction in AO activity, from 50 to 90% depending on the developmental stage. Ripe fruit (40-DAP), showed a significant increase in AO activity in wild type fruit, which was not observed in the transgenic ones, indicating a reduced AO activity also in fruit of *CoAO4* plants. Since AO is mostly located in the apoplast, AO activity was measured in the cell wall fraction of inner and outer mesocarp areas of ripe fruit, where AO is mostly active (Diallinas et al., 1997). Wild type fruit showed highest AO activity in the cell wall bound fraction of the outer mesocarp areas at the different fruit stages, whereas transgenic *CoAO4* fruit exhibited a 90% reduction in AO activity both in the inner and outer mesocarp areas (Fig. 2B). Together, these data indicate that the reduced *CmAO4* transcript accumulation observed in *CoAO4* plants drives a severe reduction of AO activity in any melon tissue studied.

The observed decrease in AO activity in the transgenic fruit could alter either the AA content and/or the AA redox state. To assess this hypothesis, we measured apoplastic AA content in melon fruit at two developmental stages, mature (32-DAP) and preclimacteric (36-DAP).

Fig. 3. Reduced AO levels affect AA biosynthetic and recycling genes. A, Expression of *CmGME*, *CmVTC2*, *CmVTC4* and *CmGaLDH* genes during fruit development. B, Expression of *CmAPX1*, *CmMDHAR* and *CmDHAR* genes during melon fruit development. Total RNA (~15 µg) from melon ovaries (ov) and fruit at different developmental stages (5-, 10-, 15-, 20-, 25-, 30-, 32-, 34-, 36-, 38-, 40-days after pollination) of Hales (WT) and *CoAO4* transgenic melon plants was fractionated in denaturing agarose gels, transferred to Hybond N-membranes and hybridized with specific ³²P-radiolabeled probes. Ribosomal RNA staining (rRNA) was used as loading control. i: inner part of the fruit mesocarp, o: outer part of fruit mesocarp.

AA content was significantly increased in the apoplast of *CoAO4* fruit, being 13- and 25-fold higher than wild type in mature and preclimacteric transgenic fruit, respectively (Fig. 2C). Moreover, DHA content was particularly low in the apoplast of *CoAO4* preclimacteric fruit, showing an 8-fold decrease compared to wild type levels (Fig. 2D). Consequently, apoplastic redox state, calculated as the ratio of AA to total ascorbate (AA + DHA), was 3- and 8-fold higher in transgenic mature and preclimacteric fruit than wild type, respectively (Fig. 2E). Together, reduction of AO activity seems to modify apoplastic AA redox state via altering both AA and DHA contents in melon fruit.

3.3. *CoAO4* melon fruit exhibit an induction in the expression of AA biosynthetic genes

The substantial reduction of AO activity and the consequent increase in AA levels in *CoAO4* fruit raised the question whether this modification influenced the AA biosynthetic and/or AA recycling pathways (Fig. 3). We first monitored the mRNA accumulation of key genes of the AA biosynthetic pathway during fruit ripening in outer and inner parts of fruit mesocarp (Fig. 3A). GDP-D-mannose 3', 5'-epimerase (*GME*) mRNA abundance remained relatively constant during wild type fruit ripening, whereas increased slightly in *CoAO4* fruit, especially in the inner mesocarp areas of ripe fruit (38- and 40-DAP; Fig. 3A).

In wild type fruit, GDP-L-galactose phosphorylase (*GGP/VTC2*) transcripts were clearly detected, mainly in the outer mesocarp of young (25- and 30-DAP) and ripe (40-DAP) fruit, while a substantial *GGP/VTC2* induction was observed in *CoAO4* fruit throughout ripening, especially in the outer mesocarp areas. L-galactose-phosphate phosphatase (*GPP/VTC4*) mRNA levels were dimly detected in wild type fruit but exhibited a significant accumulation in *CoAO4* transgenic ones during fruit development (Fig. 3A). Abundance of *GaLDH*, the last step in the AA biosynthetic pathway, increased slightly during ripening in both genotypes and exhibited higher expression in the last stages (38- and 40-DAP). Transcript accumulation of the AA recycling genes was slightly higher in transgenic fruit during ripening (Fig. 3B); in particular *DHAR* and *APX1* exhibited a substantial higher mRNA abundance in transgenic fruit during all stages of maturation (Fig. 3B), while *MDHAR* did not show critical differences. Collectively, the expression of most of the AA biosynthetic and recycling genes studied herein was induced in melon transgenic fruit and, together with AO co-suppression, they might contribute to the observed enhanced AA content.

3.4. Ripening pattern is modified in transgenic *CoAO4* fruit

Melon as a climacteric fruit is characterized by increased ethylene production rates and a rise in cellular respiration during ripening. To

Fig. 4. Reduced AO activity provoked altered ripening features. **A**, Ethylene emission rates from fruit at different development and ripening stages (30-, 32-, 34-, 36-, 38-, 40-days after pollination) of WT (white columns) and *CoAO4* transgenic melon plants (black columns). Vertical bars show standard deviations. Statistically significant differences are indicated with different letters above the bars. **B**, *CmACO* and *CmAAT1* gene expression during melon fruit development and ripening. Total RNA (~15 μg) from melon ovaries (ov) and fruit at different developmental stages (5-, 10-, 15-, 20-, 25-, 30-, 32-, 34-, 36-, 38-, 40-days after pollination) of Hales (WT) and *CoAO4* transgenic melon plants was fractionated in denaturing agarose gels and hybridized with specific ³²P-radiolabeled probes. Ribosomal RNA staining (rRNA) was used as loading control. i: inner part of the fruit mesocarp, o: outer part of fruit mesocarp. **C**, ACO activity during fruit development. Fruit at different ripening stages (30-, 32-, 34-, 36-days after pollination) of WT (white columns) and *CoAO4* transgenic melon plants (black columns) were used. Vertical bars show standard deviations. Statistically significant differences are indicated with different letters above the bars.

study whether altered AO and AA levels can affect the ripening process in melon fruit, we measured emission of ethylene throughout fruit ripening, and examined the transcript levels and activity of ethylene biosynthesis related enzymes. Altering the AA content and thus the redox state seems to provoke an earlier and a higher ethylene production in transgenic ripe fruit compared to wild type fruit (Fig. 4A), which paralleled a corresponding behavior of 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) mRNA steady state levels and ACO enzymatic activity (Fig. 4B and C).

To establish if other ripening traits were affected, we monitored the

expression of an alcohol acyl-transferase, *AAT1*, an ethylene regulated gene that plays a key role in the biosynthesis of ester volatiles and contributes to the aroma properties of ripe melon fruit (El-Sharkawy et al., 2005). *AAT1* transcripts followed the same pattern of ACO mRNAs induction and ethylene production during ripening (Fig. 4B). Although *AAT1* mRNA was detected at the same developmental stage in wild type and in *CoAO4* fruit, its accumulation was significantly higher in the latter.

Flesh firmness is a reliable indicator of fruit maturity. Increase in the AA levels or redox status caused a progressive loss in fruit firmness,

Fig. 5. Altered fruit firmness in *CoAO4* transgenic melon fruit **A**, Fruit firmness at different ripening stages (30-, 32-, 34-, 36-, 38-, 40-days after pollination) of WT (white columns) and *CoAO4* transgenic melon plants (black columns) is presented. Vertical bars show standard deviations. Statistically significant differences are indicated with different letters above the bars. **B**, Expression of *CmEXP1* and *CmMPG1* genes during melon fruit development. Total RNA (~15 µg) from melon ovaries (ov) and fruit at different developmental stages (5-, 10-, 15-, 20-, 25-, 30-, 32-, 34-, 36-, 38-, 40-days after pollination) of WT and *CoAO4* transgenic melon plants was fractionated in denaturing agarose gels and hybridized with specific ³²P-radiolabeled probes. Ribosomal RNA staining (rRNA) was used as loading control. i: inner part of the fruit mesocarp o: outer part of fruit mesocarp.

which was more evident in overripe fruit (40-DAP) (Fig. 5A). To get a better insight in the cell wall changes, we studied the expression of the cell wall modifying genes, expansin (*Exp1*) and polygalacturonase (*MPG1*) during melon fruit development and ripening. Expression of both genes appeared earlier and was more intense in transgenic *CoAO4* fruit than in wild type (Fig. 5B), similarly to the pattern of fruit firmness alteration. Taken together, all ripening physiological, physical and biochemical features studied were altered in *CoAO4* fruit, thus it appears that lowering AO levels provoke a faster and premature ripening in these transgenic melon fruit.

3.5. Silencing of AO provokes a significant reduction in fruit size

In addition to the changes observed in AA metabolism and in the ripening process, a clear effect was observed on weight and size of transgenic *CoAO4* fruit (Fig. 6A and B). Growth of transgenic *CoAO4* fruit was arrested at 32-DAP causing a significant reduction of weight in 36- to 40-DAP fruit compared to wild type (Fig. 6A and B). However, no

differences between wild type and *CoAO4* were observed during vegetative growth or in early developmental stages until 32-DAP.

The above observation led us to question whether the differences in fruit size and weight could be linked to alterations in cell number (cell division) and/or size (cell expansion) of the fruit cells, a fact that potentially could lead to the suggested link of AO function with cell growth (Pignocchi et al., 2003b). To verify this possibility, cells from inner and outer mesocarp areas of wild type and transgenic *CoAO4* fruit at 36-DAP were analyzed by confocal microscopy (Fig. 6B). This experiment was conducted in different year with higher summer temperatures that caused an earlier ripening, thus both genotypes reached their maximum size on 36-DAP. Transgenic ripe *CoAO4* fruit exhibited a greater number of cells (Fig. 6B and C), which concomitantly had smaller size both in inner and outer mesocarp areas (Fig. 6D). Taking into consideration the above results and the theories regarding AO involvement in cell-wall loosening and apoplast acidification, we hypothesized that AO may participate in cell expansion of melon fruit. This function could be related to transport processes through the plasma

Fig. 6. Effect of AO modification on fruit growth. **A**, Fruit of WT (white columns) and *CoAO4* transgenic melons (black columns) were weighted at different developmental stages. **B**, Fruit photographs of WT and *CoAO4* transgenic melons and representative confocal images of melon fruit mesocarp cross sections stained with propidium iodine. **C–D**, Fruit of the same developmental stages used to determine cell number per cross section (**C**) and cell area (**D**). **E**, PM H⁺-ATPase activity in WT (white columns) and *CoAO4* transgenic plants (black columns) 34 days after pollination in inner and outer mesocarp. Vertical bars show standard deviations. Statistically significant differences are indicated with different letters above the bars.

membrane via the stimulation of H⁺-ATPase activity (Gonzalez-Reyes et al., 1994; Kato and Esaka, 2000; Smirnov, 1996, 2000). Therefore, PM H⁺-ATPase activity was evaluated in isolated plasma membranes at inner and outer mesocarp areas from wild type and transgenic fruit at a developmental stage before the cell size reduction was observed, 34-DAP. *CoAO4* fruit, both in inner and in outer regions showed lower

PM H⁺-ATPase activity at this developmental stage when compared to wild type fruit (Fig. 6E). Indeed, the PM H⁺-ATPase activity was about 85% and 70% less at inner and outer areas in transgenic fruit, respectively. Taken together, characterization of melon *CoAO4* reveals a substantial AO participation in the rapid fruit growth typical of cucurbits, and further points out an alternative route for increasing AA content in

ripening fruit.

4. Discussion

Melons have low to medium AA content, ranging from 10 to 35 mg/100 g FW depending on morphotypes and varieties (Davey et al., 2000; Fenech et al., 2018; Mellidou et al., 2017). Despite its importance in global fruit production (over 30 million tons according to FAOSTAT), only a limited number of studies focus on the genetic manipulation of melon AA levels, and only a single AA-related QTL has been mapped on LG 5 in melon (Park et al., 2009). Therefore, the existence of different types of fruit together with the high diversity in AA content between cultivated varieties indicates that there is a great potential for improving AA levels in melon fruit. In this study, suppression of AO activity was used as an alternative route for AA bio-fortification in melon (Mellidou et al., 2017; Mellidou and Kanellis, 2017). The reduced AO activity in the *CoAO4* plants has led to a dramatic increase in the apoplastic AA content in melon fruit with a concomitant increase in the AA redox state in the apoplast. The observed correlation between low AO activity and high AA levels agrees with previous reports in tomato fruit (Abdelgawad et al., 2019; Zhang et al., 2011) and tobacco (Pignocchi et al., 2003b).

The AA pool size is regulated by the fine orchestration of biosynthesis, recycling, transport, compartmentalization and oxidation (Mellidou and Kanellis, 2017). In this regard, the changes observed in AO activity of the transgenic plants, as well as AA content and redox state, were also mirrored in alterations in transcript levels of AA biosynthetic and recycling genes. In particular, the transcript levels of *CmVTC2* and *CmVTC4* of melon fruit at the later stages of ripening were significantly higher in *CoAO4* transgenic plants compared to wild type plants, although the expression pattern during ripening showed some similarities. The increased expression of these two genes also correlated with the high levels of AA accumulation in ripe fruit, suggesting that these genes are key determinants in controlling AA biosynthesis, as previously suggested in tomato (Ioannidi et al., 2009; Mellidou et al., 2012a), kiwi (Bulley and Laing, 2016) or apple (Mellidou et al., 2012b). A less pronounced increase for *CmGME* and *CmGalDH* was observed in transgenic ripening fruit. Thus, lowering apoplastic AO activity might provoke a feed-forward amplification loop of the L-galactose biosynthetic pathway, which might further contribute to the enhancement of the AA pool size within the cells. In addition, a substantial induction of both *CmAPX1* and *CmDHAR* mRNA transcripts, and to a lesser extent of *CmMDHAR*, was recorded in transgenic ripe fruit compared to wild type ones, that might further contribute to the enhanced apoplastic and cellular AA content. The diverse effect of *MDHAR* and *DHAR* in governing fruit AA pool size has been demonstrated in different fruit species, using either QTL mapping (Mellidou et al., 2012b; Stevens et al., 2007, 2008), or gene expression analysis (Mellidou et al., 2012a). Induction of AA recycling pathway at the absence of AO activity pinpoints a feedback regulatory mechanism of AA pool size.

Previous studies have proposed that AO transcript levels are high during early fruit development in tomato, presumably due to its role in cell expansion, and then decline in ripe fruit (Ioannidi et al., 2009). By contrast, as reported herein and from other studies in melon (Diallinas et al., 1997; Moser and Kanellis, 1994; Sanmartin et al., 2007), AO expression and activity is very high in ovaries, and declines dramatically in young developing fruit followed by a transient increase in the outer mesocarp areas of ripe fruit in wild type plants. This finding probably highlights distinct roles of this enzyme in different species during the ripening process. AA is an essential molecule in ethylene production as it serves as a co-factor for the enzymatic activity of 1-aminocyclopropane-1-carboxylic acid oxidase (ACO), which catalyzes the last step of ethylene biosynthesis. In this regard, most studies have focused on the ethylene-mediated regulation of AA pool size during fruit ripening, and not *vice-versa*. Recently, ethylene has been found to regulate AA biosynthesis via ETHYLENE-INSENSITIVE3 (EIN3) and ABA INSENSITIVE4 (ABI4) in *Arabidopsis* plants, by the transcriptional repression of

VTC2 (Yu et al., 2019). Our study demonstrates that increased AA through down-regulation of AO activity can trigger an early induction of ethylene production in the transgenic fruit compared to wild-type. This is further correlated with the enhanced ACO expression and activity at the later stages of ripening. Thus, our results support that ethylene, as a key regulatory player of ripening, is transcriptionally and antagonistically modulated by AO. The cross-talk between decreased AO activity and high ethylene production can be partly explained by the enhanced availability of AA supplying H^+ to ACC in the reaction catalyzed by ACO (De Tullio, 2020) or by the alteration in the redox mediated signaling within the apoplast, as AA acts as a signaling molecule itself, independently of oxidative stimuli (Foyer et al., 2020). Another ripening-related trait, which seemed to be affected by the suppression of AO in melon is an alcohol acyl-transferase, *AAT1*, which contributes to the synthesis of volatile esters that mostly contribute to the characteristic aroma of the fruit (El-Sharkawy et al., 2005; Galaz et al., 2013). Our findings demonstrated that the expression of *AAT1* was significantly upregulated by AO reduction during ripening, probably as a result of the increased ethylene production rate.

Softening of melon fruit might be caused by the loss of hypodermal mesocarp membrane integrity (Lester and Stein, 1993). Extensive research has addressed the cell wall alterations that occur in ripening melon, indicating that cell-wall disassembly is characterized by the dismantling and re-organization of various polysaccharide networks by diverse families of wall-modifying proteins, including expansins and polygalacturonases (Nishiyama et al., 2007; Rose et al., 1998). Current knowledge on the ripening-mediated softening suggests that this process is finely regulated by alterations in pectic and hemicellulosic polysaccharides, and this process is of vital importance for the determination of commercial value of fruit crops (Wang et al., 2018). Nevertheless, it is still unclear whether this process is completely or partially hormonal regulated, whereas the involvement of AO in cell wall modifications during ripening remains largely untapped. Transgenic *CoAO4* fruit were less firm than wild type fruit, evidencing a putative function for AO in melon fruit softening. Loss of firmness was also correlated with an earlier and increased ethylene production, as well as higher transcript levels of the cell-wall modifying genes, *CmEXP1* and *CmMPG1*. Transgenic melon with reduced ACO expression has been previously shown to exert a delayed but reduced flesh firmness (Flores et al., 2001), suggesting that softening is completely or partially ethylene regulated in melon fruit. Another possible explanation for the loss of firmness in *CoAO4* fruit can be attributed to DHA, which is significantly reduced in transgenic fruit during ripening. It has been previously suggested that DHA is mostly responsible for the cell-wall loosening, either by preventing wall protein covalent cross-linking and interactions with pectin, or by actively removing calcium from cell wall by enhancing oxalate formation, resulting in a less rigid structure due to the removal of calcium from cell wall (Al-Madhoun et al., 2003; Lin and Varner, 1991). Furthermore, DHA and MDHA may facilitate vacuolation and cell-wall loosening by controlling the redox state over the cell cycle (Garchery et al., 2013). Taken together, these results support the hypothesis that AO has a direct function in fruit softening in an ethylene-dependent manner, although other ethylene-independent modes of action may also influence this complex process.

Studies on *Cucurbitaceae* and *Solanaceae* species reinforce the hypothesis that AO participates in cell expansion, as it is primarily expressed and is highly active in fast growing tissues, such as ovaries, and green immature fruit (Al-Madhoun et al., 2003; Diallinas et al., 1997; Fotopoulos et al., 2006; Pignocchi et al., 2003b; Sanmartin et al., 2007; Yamamoto et al., 2005), whereas its mode of action can vary in a species-dependent manner. AO has been mostly correlated with cell expansion early in fruit development (Kato and Esaka, 2000). However, silencing of AO in melon also indicates an AO role at later developmental stages, since a decrease in fruit size was observed in transgenic *CoAO4* ripe fruit compared to wild type. AO-mediated oxidation of AA produces MDHA, which is able to accept electrons from the plasma membrane

Fig. 7. Scheme of AA biosynthesis and recycling. A, Main ascorbate biosynthetic pathway in plants (Wheeler et al., 1998). Genes that participate in this pathway and analyzed in this work are shown in bold. B, Proposed mechanism of action for AO in cell expansion. Ascorbate transported into the apoplast by a plasma membrane associated carrier (AT) can be oxidized to MDHA by AO. Regeneration of MDHA could lead to acidification and/or hyperpolarization that stimulate vacuolization and cell loosening, thus enhancing cell expansion. The resulted MDHA is reduced back to AA by a NAD(P)H-dependent MDHAR. DHA is reduced back to AA by glutathione (GSH)-dependent DHA reductase (DHAR). This reaction generates glutathione disulphide (GSSG), which is re-reduced to GSH by NADPH-dependent glutathione reductase (GR). Adapted from Smirnoff (Smirnoff, 2000) and Villalba et al., (Villalba et al., 1996). C, Decreasing AO activity leads to a significant diminution of MDHA and DHA in the apoplast that may lower PM H^+ -ATPase activity, block hyperpolarization of plasma membrane and prevent cytoplasm and vacuole expansion. Consequently, fruit showed a decreased growth, with smaller fruit cell size.

redox system to regenerate AA in the ascorbate/glutathione cycle (Davey et al., 2000). In this case, the plasmalemma becomes hyperpolarized and a PM H^+ -ATPase is activated causing the acidification of the apoplast and activation of solute transport to cytoplasm (Gonzalez-Reyes et al., 1994). This mode of action can change the osmotic pressure causing transport of water into the cytoplasm and the vacuole, and thus cell expansion. AO silencing provokes a significant diminution of MDHA and DHA in the apoplast that may deactivate PM H^+ -ATPase (Ferjani et al., 2013) and block hyperpolarization of plasma membrane, thus preventing vacuolization and cell expansion (Fig. 7). The proposed mechanism is supported by the decreased growth, smaller cell size in the mesocarp and decreased PM H^+ -ATPase activity observed in the transgenic fruit. Thus, this is an *in vivo* evidence of the involvement of AO in cell expansion and consequently in the fast growth of cucurbits.

5. Conclusion

Our findings offer novel insights into the function of AO enzyme in melon plant. It is evident that fruit with lower AO activity not only have higher AA levels, but also exhibit an earlier induction of ripening via enhanced ethylene production, as well as smaller fruit size, accompanied by a greater number of cells and a smaller cell size. This work provides some further clues on the fine orchestration between AA oxidation and recycling in the apoplast that also implicates a crosstalk with ethylene. Taken together, our results reinforce the proposed model

for the *in vivo* AO participation in the rapid fruit growth of melon, and further point at intriguing aspects of fruit AA enhancement via the alternative route of decreasing AA catabolism that merits further exploration.

Authors contribution

AKK and MS perceived and designed the work, FC, MS, AK, GT, MSK, SV-J, TCA, SK, PT, performed the experiments, IP supervised the molecular analyses, RG supervised melon transformation, FC, MS, IM, IP and AKK designed and wrote the manuscript. All authors read and approved the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2020.08.040>.

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