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Towards a molecular strategy for improving harvesting of olives (*Olea europaea* L.)

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Abstract

Mature olive fruit that produced barely detectable levels of ethylene produced much greater quantities when they were briefly dipped in a solution containing 2 mM 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene. A single 10 s dip resulted in a transient increase in ethylene production that peaked 1 day after the application. When the fruits were dipped daily, their ethylene production remained elevated and their attachment to their pedicels weakened after 3 days.

As a first step towards producing mature olive fruit with elevated levels of ACC leading to weakened abscission zone tissue, we examined the expression of genes whose promoters might be potential candidates for driving maturation-specific expression of an ACC synthase construct in the olive. We examined the accumulation of three distinct transcripts (chalcone synthase, OE-CHS1; anthocyanidin synthase, OE-ANS1; expansin, OE-EXP1) at five stages of olive fruit development. Northern analysis showed that all three gene transcripts accumulated during ripening. Transcript abundance was lowest in green fruit, higher as the pericarp began to darken and highest at the stage where the exocarp was completely purple and the mesocarp beginning to color. None of the transcripts were detected in either young or aged leaves suggesting that they may be specific to the fruit and useful candidates for promoter isolation.

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1. Introduction

The cost of hand harvesting of olives (*Olea europaea* L.) represents more than 50% of their total production cost. Mechanical harvesting therefore has important economic advantages over traditional manual harvest. Tree shakers and other mechanical devices

have been built, but they yield only 70–80% of the fruit, depending on cultivar, maturity stage, tree size, and fruit load (Dias et al., 1999). Ethylene-releasing chemicals, used to promote olive fruit abscission, have been found to increase the efficiency of mechanical harvesting (Denney and Martin, 1994; Gerasopoulos et al., 1999). However, these compounds also cause leaf abscission, which can compromise the following year's production if more than 15–20% of the leaves are lost. A more effective means of enhancing efficiency of olive harvesting might be to localize

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production of ethylene closer to the pedicel abscission zone by enabling the fruit itself to produce ethylene at the appropriate time for harvesting. Olives are non-climacteric fruit, and ethylene production by ripening olives has been reported to be non-detectable (Rugini et al., 1982). In many tissues that produce very little ethylene, production of ethylene can be increased by exogenous application of ACC, suggesting that these tissues have the capacity to produce ethylene but do not do so due to limited production of ACC (Kende, 1993). Lack of significant ethylene production by olive fruit is likely to be due to low levels of ACC synthase in the tissue. If so, ethylene production in this fruit might be readily achieved (and controlled) by using transgenic technology to insert a functional ACC synthase construct into the olives under the control of a ripening-specific promoter.

In this study, we examined the hypothesis that ethylene production of olive fruit would be stimulated in the presence of ACC. We then isolated cDNA transcripts of three genes that we hypothesized would accumulate in maturing olive fruit and not be present in leaves. Our experiments were designed with the concept that the promoters of such genes would be candidates for driving the ripening-specific expression of an ACC synthase construct in transgenic olive fruit.

2. Material and methods

2.1. Plant material

Fruiting branches of olives (*Olea europea* L. cv. Manzanillo) approximately 40 cm in length were harvested in January from a local orchard, and immediately transported to the postharvest laboratory. The branches were recut and their bases placed in a solution of 50 ml l⁻¹ NaOCl (to prevent growth of microbes that might prevent hydration). Conditions in the postharvest laboratory were 20 ± 2 °C, relative humidity of ca. 55% and a 12 h photoperiod (15 μmol m⁻² s⁻¹ PAR from Cool White fluorescent lamps).

2.2. ACC application, ethylene production, and removal force

All the Stages 4 and 5 fruit attached to harvested branches were dipped once (first experiment), or

daily (subsequent experiments), either in water or in 2 mM 1-aminocyclopropane-1-carboxylic acid (ACC, Sigma, St. Louis, MO) for ca. 10 s. Triplicate branches were used for each treatment. The force required to detach the pedicel from three randomly selected fruit from each branch was measured daily for up to 6 days with a locally-constructed electronic force gauge. After the force measurements were made, the fruit from each treatment were placed in sealed glass jars for ethylene determination. Ethylene evolved from the fruit after 1 h was measured using a Carle AGC 111 gas chromatograph (Fullerton, CA, USA) fitted with a 1 m activated alumina column and operating at 70 °C.

2.3. Stages of olive fruit development and anthocyanin content

Olives were harvested from a tree with fruit at diverse stages of maturity and separated into five maturity classes (Stage 1, green exocarp/green mesocarp; Stage 2, slightly reddened exocarp/green mesocarp; Stage 3, half purple exocarp/green mesocarp; Stage 4, fully purple exocarp/slightly purple mesocarp; Stage 5, fully purple exocarp/purple mesocarp). The pits were removed and combined samples of exocarp and mesocarp from fruit at the different maturities were frozen in liquid N₂ and stored at -80 °C until needed. Samples of the frozen tissue were extracted into methanolic HCl following the standard AOAC method (Harborne, 1967) and the concentration of cyanidin-3-glucoside equivalents determined spectrophotometrically at 535 nm.

2.4. RNA isolation

Total RNA was extracted from olives at the five defined stages of maturity using a modification of the hot borate technique (Wan and Wilkins, 1994) as outlined in Hunter et al. (2002). RNA was determined spectrophotometrically at 260 nm.

2.5. Amplification of anthocyanidin synthase, chalcone synthase, and expansin sequences

Total RNA was isolated from Stage 4 olive fruit and 5 μg used as a template for first strand cDNA synthesis using 50 pmol of 5'-CGGTACCGATAAGCTTGAT-(18)V-3' and 200 U SuperscriptIITM (Invitrogen/

Gibco-BRL, Carlsbad, CA). Putative anthocyanidin synthase (ANS), chalcone synthase (CHS), and expansin (EXP) sequences were amplified from the cDNA by PCR using the following primers: ANS sense 5'-AGCAAGTTHGCMAAYARTGC-3' and antisense, 5'-CGGTACCGATAAGCTTGA-3' (Rosati et al., 1999); EXP sense 5'-YTGCCARTTYTGCC-CCARTT-3' and antisense 5'-GSNCAYGCNACNT-TYTAYGGNG-3' (Rose et al., 1997); CHS sense, 5'-GACATGCCTGGYGCYDAYTAYCA-3' and antisense 5'-ACRCATGCRCTTGACATGTTTCCRTA-3'. PCR was carried out using HotstarTaq (Qiagen, Valencia, CA) according to the instructions of the manufacturer. PCR conditions were: 95 °C at 15 min, then 40 cycles at 94 °C for 1 min, 50 °C for 1.5 min and 72 °C for 1.5 min with a final extension at 72 °C for 7 min. Following amplification the products of expected sizes (ANS, ca. 800 bp; CHS, ca. 650 bp; EXP, ca. 500 bp) were gel-purified and cloned into *E. coli* following the instructions of the pGEM[®]-T Easy Vector System II kit (Promega, Madison, WI). Cloned products were sequenced on an automated DNA sequencer (Model 377, PRISM, Applied Biosystems, Foster City, CA) and identified using the BLASTx algorithm (Altschul et al., 1990).

2.6. Northern analysis

Denatured RNA was separated at 100 V for 3–5 h in 1% (w/v) agarose gel containing 0.22 M formaldehyde and transferred to a Hybond[™]-N+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ) as described by Ingelbrecht et al. (1998).

Probes were prepared by amplifying the cloned ANS, CHS, and EXP inserts from their plasmids and radiolabelling the gel-purified products with ³²P dATP according to the instructions of the random primed Strip-EZ[™] DNA labeling kit (Ambion, Austin, TX). The radiolabelled sequences were then purified through ProbeQuant micro-columns (Amersham Pharmacia Biotech), denatured, and added to the Ultrahyb[™] ultrasensitive hybridization buffer (Ambion) bathing the RNA containing membranes at 42 °C. After approximately 16 h, the membranes were washed in 2 × SSC (0.3 M NaCl/3 mM sodium citrate pH 7.2/0.1% (w/v) SDS) at 65 °C for 15 min followed by a further two washes in 1 × SSC/0.1% SDS and 0.1 × SSC/0.1% SDS. Membranes were

then sealed in plastic bags and the hybridized label detected using the Storm[®] Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Blots were stripped following the instructions in the Strip-EZ[™] DNA labeling kit manual.

3. Results

3.1. ACC application and ethylene production of olive fruit

A single topical application of 2 mM ACC to Stages 4 and 5 olive fruit caused a substantial but transient increase in ethylene production (Fig. 1). Production increase became 17-fold higher than controls 1 day after the application, but by day 3, levels of ethylene produced were back to those of the controls. When the fruit were dipped in a solution containing 2 mM ACC for ca. 10 s every day, ethylene production remained high throughout the experiment and ranged from 3 to 7 nl g⁻¹ FW h⁻¹ (Fig. 2).

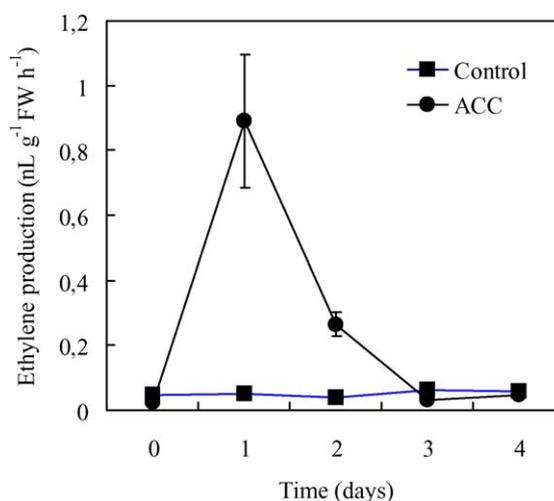


Fig. 1. Ethylene production from olive fruit given a single topical application of ACC. The fruit attached to harvested branches were dipped once either in water or in 2 mM 1-aminocyclopropane-1-carboxylic acid (ACC). Each day, selected fruit from the branches were detached and placed in sealed glass vials and the amount of ethylene that accumulated in the headspace was determined after 1 h by gas chromatography. Data are the mean and standard error of four independent measurements. The trend was repeated in three independent experiments.

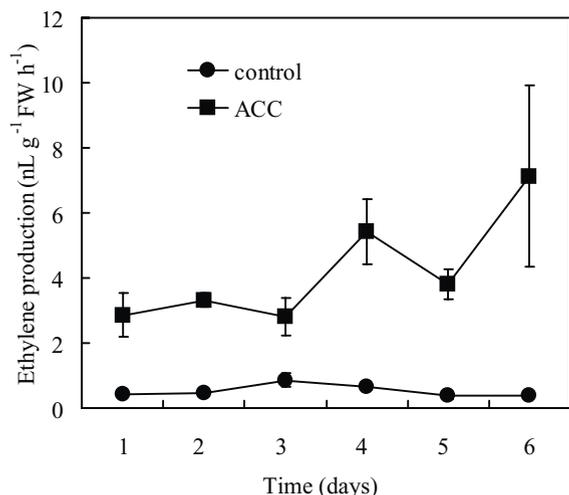


Fig. 2. Ethylene production from olive fruit dipped daily in ACC. Fruit on six replicate branches were dipped daily in water or in 2 mM ACC. Each day, one to three fruit were detached from each branch and were placed in sealed glass vials. The ethylene evolved by the fruit during 1 h was measured by gas chromatography.

3.2. ACC application and detachment force of olive fruit

The force required to detach the fruit from its pedicels remained high in control fruit throughout the experiment (Fig. 3). In contrast, the detachment force for olives treated daily with ACC began to decrease

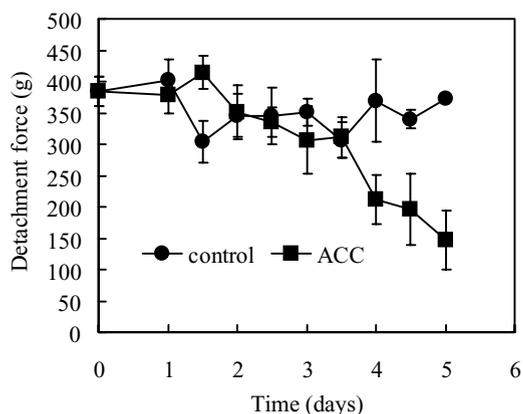


Fig. 3. Effect of ACC treatment on the pull force required to detach olive fruit from their pedicels. Fruit on six replicate branches were dipped daily in water or 2 mM ACC and each day the force required to detach the pedicel of one to three fruit taken from each branch was measured using a force gauge.

rapidly and linearly after 3 days, and by 4–5 days some of the fruit were abscising spontaneously. We did not notice any effect of applying ACC to fruit on leaf abscission.

3.3. Changes in anthocyanin content of maturing olives

The level of cyanidin-3-glucoside equivalents in olive fruit increased substantially as the fruit matured (Fig. 4A). In ripe fruit, the anthocyanin content was three-fold that present in the green fruit.

3.4. Isolation of ripening-associated genes

Reverse transcriptase-polymerase chain reaction (RT-PCR) with the CHS primer set amplified a 571 bp fragment (OE-CHS1, accession number AF384049) that showed 92% amino acid identity over its entire length to the naringenin-chalcone synthase from snapdragon. The OE-CHS1 cDNA recognized a transcript on the Northern blot of approximately 1.4 kb. The ANS primer set amplified a 790 bp fragment (OE-ANS1, accession number AF384050), of which 650 bp encoded the 3'-end of the coding frame and the remaining 140 bp the 3'-UTR. The 650 bp coding portion showed 90% identity at the amino acid level to an anthocyanidin synthase from *Forsythia × intermedia*. The OE-ANS1 sequence hybridized to a transcript of approximately 1.3 kb on the Northern blot. The EXP primer set amplified a 488 bp cDNA fragment (OE-EXP1, accession number AF384051) that showed 88% identity over its entire length to expansin 2 of *Zinnia elegans*. The OE-EXP1 sequence hybridized to an ca. 1.2 kb RNA on Northern blots.

3.5. Accumulation of chalcone synthase, anthocyanidin synthase, and expansin transcripts during ripening of olive fruit

OE-CHS1, OE-ANS1, and OE-EXP1 transcripts accumulated similarly in the olive fruit as it matured (Fig. 4B). Abundance was lowest in Stage 1 (green exocarp/green mesocarp) fruit and highest in Stage 4 (fully purple exocarp/purplish mesocarp). None of the transcripts showed detectable hybridization to RNA isolated from the leaves.

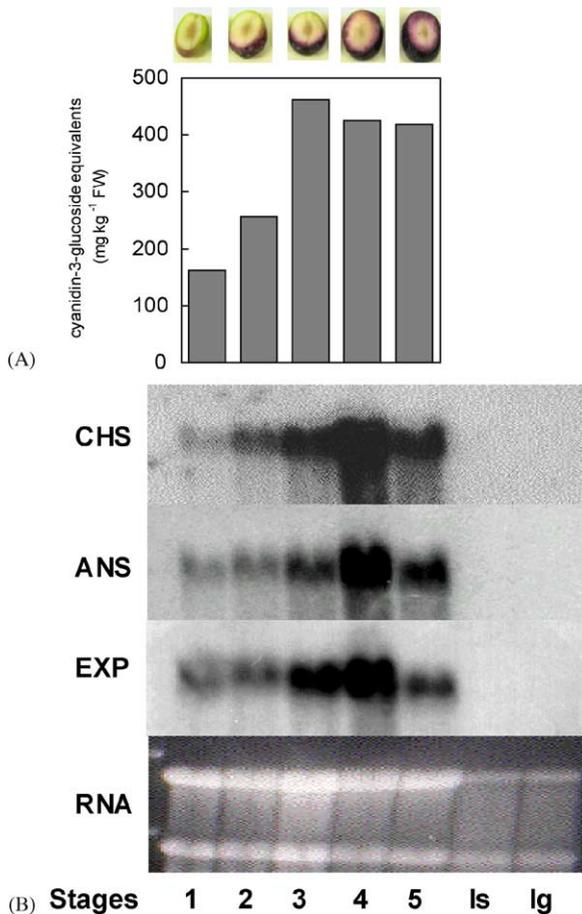


Fig. 4. Abundance of gene transcripts encoding chalcone synthase (CHS), anthocyanidin synthase (ANS) and expansin (EXP) in the olive pericarp during fruit development and in green (lg) and yellowing leaves (ls). Fruit at five stages of maturation were harvested, their anthocyanin content determined (A) and their total RNA content isolated. Twenty micrograms of total RNA from each fruit maturation Stages 1–5 and from green and yellowing olive leaves was separated by electrophoresis and blotted onto HybondTM-N⁺. The blots were probed with ³²P dATP radiolabelled cDNA sequences encoding CHS, ANS, and EXP (B).

4. Discussion

Now that transformation procedures for olives have been developed (Rugini and Caricato, 1995), a strategy such as that outlined in this manuscript could be used to solve the problem of the high cost of hand harvesting. Olive fruit, like strawberry (Tian et al., 1997), normally produces negligible amounts of ethylene throughout maturation, but as with strawberry

(Perkins-Veazie et al., 1987) produces ethylene after application of ACC to the surface of the fruit. This suggests that ethylene production is blocked because of limited amounts of ACC in the tissue, most probably due to limiting amounts of ACC synthase (Yang and Hoffman, 1984).

ACC synthase (EC 4.4.1.14) is widely regarded as the major rate limiting enzyme of ethylene biosynthesis in higher plant tissue (Kende, 1993). The enzyme is normally present in vanishingly low concentrations, is labile and undergoes catalytic-based inactivation (Acaster and Kende, 1983; Bleecker et al., 1986; Satoh and Esashi, 1986). These aspects are all consistent for an enzyme involved in controlling a biosynthetic pathway and for an enzyme that could effectively be controlled at the level of transcription. In this regard, there are many instances where ethylene production rates positively correlate with ACC synthase transcription (Sato and Theologis, 1989; Huang et al., 1991; Olson et al., 1991; Harpster et al., 1996).

In climacteric fruit, ACC synthase transcript abundance and activity increases significantly during ripening (Yip et al., 1992). In this fruit, a single application of ACC or ethylene causes sustained transcription of ACC synthase and production of ethylene. However, in olives, ACC synthase is apparently not induced by ACC or ethylene since a single application of ACC to the olive fruit caused only a transient increase in ethylene production. The ethylene production stopped, presumably because of depletion of the exogenously-added ACC.

Although olive fruit produces negligible amounts of ethylene, like strawberries (Perkins-Veazie et al., 1987; Luo and Liu, 1994), they still respond to the presence of the hormone. According to Rugini et al. (1982), the timing of the ethephon (ethylene-releasing) treatment determines whether ethylene affects ripening of olives. Ethephon has a marked effect on ripening (early pigmentation, increased fructose content), when applied before the olives respire at their maximum rate. Applying ethephon to the fruit after the maximal respiration rate results only in weakening of the pedicel. In this study, we applied ACC to attached fruit on branches just prior to normal harvest time and were able to measure a weakening in the attachment of the pedicel to the olive fruit within 3 days. This is presumably a response to the ethylene formed from ACC because the weakening occurs well after the

ACC-induced increase in ethylene production of the tissue. Ethephon has previously been used to improve shake harvesting of olives, but results in undesirable leaf abscission, which is detrimental to the following years' harvest. We treated the fruit directly with ACC to simulate natural production of ethylene by the fruit and did not observe increased leaf fall with this treatment. Since the control fruit produced very low levels of ethylene, it seems probable that the detached branchlets that we used represent a reasonable model for behavior on the tree, and that there was no effect of detachment on the synthesis or response to ethylene.

Given that olive fruit has the ability to convert ACC to ethylene in sufficient amounts to weaken the attachment of the pedicel/fruit attachment, and that transformation of olives is being achieved (Rugini et al., 1982), it should be possible to produce a transgenic olive tree with ACC synthase expressed specifically in the fruit at the correct maturation stage to improve the harvesting efficiency of olive fruit. In this manuscript, we demonstrated that transcripts encoding proteins involved in fruit pigmentation (chalcone synthase and anthocyanidin synthase) and possibly fruit softening (expansin; Brummell et al., 1999) accumulate in ripening olive fruit, but not in leaves. If further investigation demonstrates that these genes are uniquely up-regulated in ripening fruit (and not in other tissues or at other stages of development), their promoters may be candidates for driving the expression of ACC synthase in ripening olive fruit.

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