α -L-Arabinofuranosidase Activity during Development and Ripening of Normal and ACC Synthase Antisense Tomato Fruit

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Abstract. α -L-Arabinofuranosidases (α -Af) are plant enzymes that have the capacity to release terminal arabinofuranosyl residues from a wide variety of pectic and hemicellulosic polymers, as well as different glycoconjugates. Our interest in α -Af is related to its potential role in ripening-related loss of arabinose from tomato fruit cell walls. Using both control (cv. VF 36) and ACC synthase antisense (A11.1) tomatoes (*Lycopersicon esculentum* Mill.), we demonstrate that tomato α -Af activity is present during the entire ontogeny of the fruit. Immature 10-day-old fruit displayed 6-fold more α -Af activity on a per gram fresh weight basis, than mature green fruit. In VF 36 fruit, α -Af activity increased 45% from mature green 4 (48 days post anthesis) to light red stages (55 days) when fruit ripened on the vine. In contrast, no similar increase was detected in ACC synthase antisense fruit that do not ripen in the same time frame. However, when A11.1 fruit were detached at 48 days after anthesis and treated continuously with 100 mL·L⁻¹ ethylene the fruit ripened and α -Af increased, as in ripening normal fruit. The α -Af activity pattern is similar to that reported for tomato β -galactosidases. The increasing α -Af activity during ripening and the decreased activity in antisense ACC synthase fruit after reaching the mature green stage suggest a role for ethylene in the ripening-related synthesis or activation of this enzyme.

Texture is a major attribute that has a strong effect on consumer perception of tomato fruit quality. Different factors affect tomato textural properties, among them cell wall polysaccharide composition (Barrett et al., 1998). Most of the covalent modifications in cell wall polysaccharides result from the activity of a set of hydrolases that may participate in a concerted enzymatic action (Fischer and Bennett, 1991). A substantial decrease in cell wall-bound galactosyl and arabinosyl residues is one of the most evident cell wall compositional changes during fruit ripening (Gross and Sams, 1984; Seymour et al., 1990). These neutral sugar components usually occur as side chains (5-arabinan, 4-

galactan, arabinogalactan) attached to rhamnosyl residues of the rhamnogalacturonan backbone (Carpita and Gibeau, 1993). Terminal α -L-arabinosyl units are also present in the arabinoxyloglucans of the Solanaceae (York et al., 1996) and in the substantial carbohydrate component of arabinogalactan proteins (AGPs; Cassab, 1998 and references cited therein).

β -galactosidases in growing and ripening tomatoes have been studied in relationship to their potential for removing galactosyl residues from cell wall polymers (Carey et al., 1995; Carrington and Pressey, 1996; Pressey, 1983; Sozzi et al., 1998a) and their impact in tomato fruit metabolism and softening is now being investigated using transgenic plants (Smith and Gross, 2000; Smith et al., 1998). However, the loss of cell wall arabinosyl residues has received much less attention. Cell wall arabinose loss continues after harvest in mature *rin* tomato fruit even though they do not soften (Gross and Wallner, 1979). However, there have been no studies of the effect of the specific suppression of ethylene synthesis on cell wall arabinose change to date.

The cell wall is by far the major arabinose-containing structure of plants. Arabinose is the primary neutral sugar residue lost, during maturation, in some commercially important fruits, such as pears (*Pyrus communis* L.), peaches and nectarines [*Prunus persica* (L.) Batsch], blueberries (*Vaccinium corymbosum* L.) and strawberries (*Fragaria × ananassa* Duch.) (Gross and Sams, 1984) and significant changes in arabinose content have also been detected in avocado (*Persea americana* Mill.) (Redgwell et al., 1997). In tomato, the most intensively studied fruit over the last decades, 25% of the cell wall arabinose may be released in the 4- to 5-d period between the turning and the red ripe stages (Gross and Sams, 1984). Several polysaccharide types might be involved. Limited breakdown of xyloglucan occurs early in tomato fruit ripening and pectins are hydrolyzed increasingly as fruit become red ripe (Brummell et al., 2000). The only report of AGP metabolism during tomato fruit development describes accelerated synthesis of AGP carbohydrate early in ripening (Huysamer et al., 1997).

Fruit softening is not the only reason to study the enzymes potentially capable of removing arabinosyl residues. The arabinogalactan proteins may be involved in important processes during growth and development, including modifications of cell wall composition and cell-to-cell associations (Cassab, 1998). Moreover, Priem et al. (1993) reported a tomato protein-associated *N*-glycan containing an arabinosyl residue and suggested that *N*-glycans could have a key role in the regulation of tomato fruit senescence. Thus, the mechanisms responsible for the release of arabinosyl residues could also modulate ripening-related biological processes in addition to softening.

α -L-Arabinofuranosidase [α -L-arabinofuranoside arabinofuranohydrolase, EC 3.2.1.55; α -Af] has been widely studied in microorganisms (e.g., Saha, 2000) and plant tissues (e.g., Konno et al., 1987). It has also been detected in several fruits [e.g., Japanese pear (*Pyrus serotina* Rehder) Tateishi et al., 1996; goldenberry (*Physalis peruviana* L.); Trinchero et al., 1999], and in both the pericarp (Campbell et al., 1990; Sozzi et al., 1998b) and locule (Cheng and Huber, 1997) cell walls of tomato. This paper describes work examining the presence of α -Af during tomato growth and ripening and uses ACC synthase antisense fruit to determine whether ethylene influences α -Af activity during ripening.

Materials and Methods

Plant material and chemicals. Control tomato seeds (cv. VF36) and transgenic seeds (called A11.1, which are in the 'VF36' genetic background) expressing antisense ACC-synthase RNA were obtained from Athanasios Theologis [Plant Gene Expression Center, Univ. of California–Berkeley, U.S. Dept. of Agriculture (USDA), Albany, Calif.]. Forty plants of each type were grown under daylight in 15-L plastic pots in a greenhouse at the Univ. of California, Davis. Tomato plants

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were trellised and fertilized as described by Cadahía López (1995). Flowers of control and antisense ACC synthase plants were tagged at anthesis. Both control and antisense fruit were harvested at different phenology stages. Only the early-set proximal fruit of each truss were selected to minimize alterations due to different hormonal fluxes and variations of assimilate import. Forty-eight-day-old (mature green stage) control and antisense fruit were harvested and stored at $20 \pm 1^\circ\text{C}$ in humidified air and diffuse light until used. Ripeness stages in normal 'VF36' fruit were established in comparison with the tomato color chart of the USDA (1976), as follows: mature green 4 (day 48); breaker (day 50); light red (day 55); red ripe (day 57); over ripe (day 70). Untreated antisense fruit do not ripen and so comparisons with ripening VF36 fruit are based on fruit age (days after anthesis, DAA). Control fruit could be utilized up to 80 DAA; samples after that date were judged not to be marketable due to excessive softening. A sample of 48 DAA antisense fruit was enclosed in 4-L glass jars (2 tomatoes per jar) and exposed to a mixture of $100.0 \pm 0.5 \mu\text{L}\cdot\text{L}^{-1}$ ethylene in humidified air utilizing a constant flow-through gas system. The desired ethylene concentration was reached within 1 h after placing the fruit into the containers and was maintained throughout the imposed ripening period. The flow rate (1100 mL/min) selected ensured that CO_2 accumulation would not exceed 0.2 kPa; this was checked once a day with an infrared CO_2 analyzer (model PIR-2000R; Horiba Instruments, Irvine, Calif.). Defect-free fruit were rinsed with distilled water and dried with paper towels. All chemicals were from Sigma Chemical Co. (St. Louis).

Enzyme extraction and assay. Triplicate composite pericarp samples (25–100 g, depending on the fruit stage) were homogenized in a Waring blender (45 s) and then by using a Polytron (45 s) with 3 vol of cold 100 mM sodium acetate buffer (pH 5) containing 1.4 M NaCl, 1 mM ZnCl_2 , 5 mM 2-mercaptoethanol, and 1.5% (w/v) PVPP. The subsequent steps were performed at 4°C . The suspension was stirred for 30 min, centrifuged at $12000 g_n$ for 15 min, and filtered through glass filter paper (GF/C, Whatman).

Aliquots of filtered extract were assayed for total α -Af activity using *p*-nitrophenyl- α -D-arabinofuranoside as substrate. Reaction mixtures contained 250 μL of 0.1 M citrate buffer, pH 4.5, 200 μL of 0.1% bovine serum albumin, 50 μL of enzyme solution and 200 μL of 13 mM substrate solution, with incubation at 37°C . The generation of free *p*-nitrophenol is linear for 3 h using these reaction conditions. Activities reported are based on rates determined after 1 h with the reaction stopped by addition of 1 mL of 0.13 M sodium carbonate. Absorbance was measured at 400 nm. Free *p*-nitrophenol was used as standard.

Results and Discussion

Preliminary tests showed that the extractability of α -Af was salt dependent. The requirement for high NaCl in the extraction

medium suggests that α -Af is bound to or at least tightly associated with the cell walls. Tateishi et al. (1996) have reported that extraction of Japanese pear α -Af was enhanced using an extraction buffer containing high LiCl, as opposed to NaCl, concentration. However, this approach was not useful for extracting the tomato α -Af activity (15% to 20% of the total activity extracted using NaCl). This low recovery of activity is probably due to the instability of the tomato α -Af when exposed to dialysis (Sozzi et al., 1998b). Inclusion of the chelators ethylenediamine- tetraacetic acid and trans-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid in the extraction buffer did not significantly improve solubilization capacity of the extraction medium.

Previous reports (Campbell et al., 1990; Sozzi et al., 1998b) have indicated that there is little α -Af activity in tomato fruit tissues. The protocol used for this experiment strongly enhanced α -Af activity recovery, due to inclusion of Zn^{2+} (activation, stabilization effect, or both), PVPP (removal of phenolic compounds), and 2-mercaptoethanol (maintenance of possible sulfhydryl groups within the active site region in a reduced state) in the extraction medium and the homogenization with a Polytron. The Zn^{2+} enhancement of α -Af activity recovery was generally 10% to 15% in pericarp crude extracts.

Our results indicate the presence of α -Af early in the development of both control and antisense ACC synthase tomato fruit, with no differences in activity between the two lines (Fig. 1). There are many reports of relatively high activities of a variety of glycosidases early in development of several fruits (e.g., studies with tomato α - and β -galactosidases, Smith and Gross, 2000; Sozzi et al., 1998a). High activities of α -Af and other putative wall-modifying enzymes may reflect functions in wall synthesis and assembly or wall

component breakdown to facilitate cell division or growth, or may be unrelated to fruit cell wall metabolism. Early in fruit development, α -Af may be modulated by gibberellins or auxins, hormones which tend to be at relatively high concentration at this time (Mapelli et al., 1978). Exogenous IAA and GA_3 have been shown to increase α -Af activity in intact breaker tomatoes (Sozzi et al., 2000). High α -Af activity has also been reported for Japanese pear fruit (Tateishi et al., 1996) during the early stages of development. α -Af activity was detected throughout fruit development. Activity declined on a fresh weight basis during the cell expansion phase until maximal fruit size was reached, but total fruit activity increased steadily until 35 DAA and then decreased slightly as the mature green stage was reached (Fig. 1).

During ripening, control fruit expressed steadily increasing levels of α -Af activity as the fruit matured to the red ripe stage (Fig. 2) suggesting a role for α -Af in the cell wall arabinose loss that parallels fruit softening. In contrast, the α -Af activity in antisense fruit decreased after the mature green stage (48 DAA), continuing to fall as the fruit failed to ripen (based on red color development; see Sozzi et al., 1998a). The first color change was seen at the blossom end 32 d after harvest (80 DAA), and α -Af activity remained low. Application of $100 \mu\text{L}\cdot\text{L}^{-1}$ ethylene to mature green ACC-S antisense tomatoes caused an increase in α -Af activity (Fig. 2). It is well known that initiation and progress of climacteric fruit ripening requires the presence of ethylene and that tomato ripening is associated with cell wall-hydrolase increases (Fischer and Bennett, 1991). Normal ripening fruit and the antisense fruit that have been treated with ethylene show substantial increases in α -Af. Therefore, we conclude that the absence of an increase in α -Af activity in antisense toma-

Fig. 1. Total α -L-arabinofuranosidase activity and fruit fresh weight during the growth of normal and ACC synthase antisense tomato fruit. Values represent the mean $\pm$ SD ($n = 3$).

Fig. 2. Total α -L-arabinofuranosidase activity during ripening of normal and antisense tomato fruit. Values represent the mean $\pm$ SD ($n=3$). Ripeness stages in normal 'VF36' fruit were established in comparison with the tomato color chart of the USDA (1976), as follows: mature green 4 (day 48); breaker (day 50); light red (day 55); red ripe (day 57); over ripe (day 70). Ethylene treatment of antisense fruit causes them to pass through ripeness stages in the same time frame as normal fruit.

atoes, after they reach the mature green stage, reflects the reduction of ethylene synthesis and, subsequently, ethylene action. These results allow us to suggest a role for ethylene in the expression of α -Af gene(s), as probably happens with the genes encoding the ripening-related enzymes polygalacturonase (Sitrit and Bennett, 1998) and β -galactosidase II (Smith and Gross, 2000; Smith et al., 1998). Alternatively, ethylene could influence translation of a stable α -Af mRNA or activation of an α -Af preprotein.

The α -Af activity pattern throughout tomato fruit ontogeny is very similar to that of β -Gal (Sozzi et al., 1998a). For that activity, several isoforms, present at various stages through fruit development, have been identified. However only one ethylene-regulated isoform participates in ripening-related cell wall galactose metabolism (Carey et al., 1995; Carrington and Pressey, 1996; Pressey, 1983; Smith and Gross, 2000; Smith et al., 1998; Sozzi et al., 1998a). This similarity suggests that tomatoes also have more than one α -Af isoform and this is the subject of continuing investigation. Of particular interest would be demonstrations that α -Af (perhaps a specific isoform) acts on tomato cell wall substrates and that α -Af presence correlates with the cell wall arabinose loss that accompanies normal fruit ripening. The antisense ACC synthase fruit could be an excellent system in which to make these tests.

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