Gibberellic Acid, Synthetic Auxins, and Ethylene Differentially Modulate α-L-Arabinofuranosidase Activities in Antisense 1-Aminocyclopropane-1-Carboxylic Acid Synthase Tomato Pericarp Discs[1]

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α-L-Arabinofuranosidases (α-Afs) are plant enzymes capable of releasing terminal arabinofuranosyl residues from cell wall matrix polymers, as well as from different glycoconjugates. Three different α-Af isoforms were distinguished by size exclusion chromatography of protein extracts from control tomatoes (*Lycopersicon esculentum*) and an ethylene synthesis-suppressed (ESS) line expressing an antisense 1-aminocyclopropane-1-carboxylic synthase transgene. α-Af I and II are active throughout fruit ontogeny. α-Af I is the first Zn-dependent cell wall enzyme isolated from tomato pericarp tissues, thus suggesting the involvement of zinc in fruit cell wall metabolism. This isoform is inhibited by 1,10-phenanthroline, but remains stable in the presence of NaCl and sucrose. α-Af II activity accounts for over 80% of the total α-Af activity in 10-d-old fruit, but activity drops during ripening. In contrast, α-Af III is ethylene dependent and specifically active during ripening. α-Af I released monosaccharide arabinose from KOH-soluble polysaccharides from tomato cell walls, whereas α-Af II and III acted on Na_2CO_3-soluble pectins. Different α-Af isoform responses to gibberellic acid, synthetic auxins, and ethylene were followed by using a novel ESS mature-green tomato pericarp disc system. α-Af I and II activity increased when gibberellic acid or 2,4-dichlorophenoxyacetic acid was applied, whereas ethylene treatment enhanced only α-Af III activity. Results suggest that tomato α-Afs are encoded by a gene family under differential hormonal controls, and probably have different in vivo functions. The ESS pericarp explant system allows comprehensive studies involving effects of physiological levels of different growth regulators on gene expression and enzyme activity with negligible wound-induced ethylene production.

Fruit differentiation, growth, and ripening depend on changes in the architecture of cell walls. These processes involve the modification of the amount and composition of pectic and hemicellulosic polysaccharides, which takes place as a coordinated series of assembly and disassembly steps. The removal of side chains from the backbones of different matrix polysaccharides is attributable to the action of glycosidases (Fry, 1995), but the actual role of these enzymes in vivo and their regulation remain unknown. In the last few years, considerable attention has been given to the release of neutral sugars from the cell wall, a major process in the development and ripening of tomato (*Lycopersicon esculentum*) fruit, and to understanding how the various enzymes and their different isoforms affect these processes. A multigenic β-galactosidase (β-Gal) gene family has recently been identified (Smith and Gross, 2000); three different gene products were purified and characterized for the first time two decades ago (Pressey, 1983). One of them, designated β-Gal II, is specifically active during ripening and is probably involved in fruit softening (Pressey, 1983; Carey et al., 1995; Smith et al., 1998; Sozzi et al., 1998). However, little is known about other major glycosidase classes, including the arabinofuranosidases.

α-L-Arabinofuranosidases (α-Afs; α-L-arabinofuranoside arabinofuranohydrolases, EC 3.2.1.55) catalyze the hydrolysis of terminal nonreducing α-L-arabinofuranosyl residues from various pectic and hemicellulosic homo- (arabinans) and heteropolysaccharides (arabinogalactans, arabinoxylans, arabinoxyloglucans, glucuronoarabinoxylans, etc.) as well as from different glycoconjugates (Beldman et al., 1997; Saha, 2000). We have reported that α-Af activity is detectable throughout preripening development of control and ethylene synthesis-suppressed (ESS; antisense 1-aminocyclopropane-1-carboxylic acid [ACC] synthase transgene-expressing) fruit, but that the large increase in the extractable α-Af activity exhibited by ripening control fruit only occurred in the ESS fruit if they were given postharvest ethylene treatment (Sozzi et al., 2002). These results suggested the presence of more than one isoform during growth and ripening.

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Different approaches have been made to understand growth regulator relationships and their influence on fruit during development and ripening, but different problems have hindered research in this area (Brady, 1987). Studies with whole tomato fruit require vacuum infiltration of different regulators or a long-time dipping of the fruit. Thus, entry and distribution of a growth regulator are uncertain due to surface diffusion barriers, and the treatment of each fruit is different depending on its anatomy and morphology. In an alternate manner, dipping fruits in concentrated hormonal solutions, often 10^{-5} M or higher, induces ethylene formation as a stress response mediated by an enhanced ACC synthase activity (Vendrell, 1988; Sozzi et al., 2000). Cohen (1996) cultured immature tomato flowers to generate fruits with slowed ripening following treatment with indole-3-acetic acid. Tomato calyx and fruit cultures have been used to characterize metabolic aspects of ripening, including ethylene synthesis (Ishida et al., 1993; Ishida, 2000). Although these studies have provided useful insights into hormonal control of development, the entire course of development proceeded in the absence of seeds, a factor that might have influenced aspects of the ripening process. Another possible approach that affords the opportunity of adding a substance uniformly to fruit pericarp tissue is the use of excised discs. However, the slicing of wild-type tomatoes causes the production of wound-induced ethylene by excised discs (Campbell et al., 1990). Thus, it could be argued that changes in the activity of specific enzymes may be influenced by the wound-induced ethylene as well as the hormone treatment or, alternatively, by both.

Inhibition of the expression of ACC synthase, achieved in transgenic tomato plants (Oeller et al., 1991), is particularly useful in assessing whether ethylene enhances or triggers the gene expression or the activity of potential cell wall-modifying enzymes (Sitrit and Bennett, 1998; Sozzi et al., 1998) without applying inhibitors of ethylene synthesis or action. Herein, we demonstrate the utility of a pericarp disc system from ESS fruit for testing fruit tissue responsiveness to physiological levels of several biological regulators, including ethylene. We report on a family of at least three different α-Afs and we show that α-Af isoform activity in hormone-treated discs is consistent with that measured during intact tomato fruit ontogeny, thus providing support for the idea that the α-Af family members are responsive to different endogenous controls during growth and ripening.

RESULTS AND DISCUSSION

Three α-Af Isoforms Are Present during Tomato Fruit Ontogeny

When a Sephacryl S-200 size-exclusion chromatography (SEC) column was equilibrated and eluted with 100 mM sodium acetate, pH 5, containing 1 M NaCl and 1% (w/v) Suc, three peaks of α-Af activity were obtained. They were designated α-Af I, II, and III (Fig. 1; Table I). When the column was run at pH 6, a different peak (designated peak A) was recovered from the SEC fractions (Fig. 2). After pH adjustment and concentration, peak A was rechromatographed at pH 5 and peak III was restored (compare Figs. 1 and 2), suggesting the occurrence of a disaggregation/aggregation process.

The three isoforms were bound by concanavalin A-agarose, thus suggesting that these enzymes are glycoproteins. The activity was recovered when the isoforms were eluted with methyl-α-D-mannopyranoside (MM; Table I). Methyl-α-D-arabinofuranoside (MAf) was synthesized (Ness and Fletcher, 1958) and used as a selective eluant for the α-Af bound to concanavalin A-agarose (Matheson and Saini, 1977). However, although protein was eluted with the MAf, the inhibition of α-Af activity assays by MAf (data not shown), combined with the loss of α-Af activity that often occurs upon dialysis (see below), prevented the use of MAf as a specific eluant for α-Af purification.

When the active fractions from the SEC column were collected and dialyzed, most of the enzyme activity was lost (Fig. 3). Further purification by standard methods (e.g. ion-exchange chromatography and hydrophobic resins) resulted in the inactivation of the enzyme. Total α-Af also proved to be unstable in crude extracts stored at −20°C, with 70% of the activity lost over 60 d. The addition of trehalose (10%, w/v) plus Suc (1%, w/v) as stabilizers partially counteracted the loss of activity, reducing it by about one-half. When added separately, trehalose was a better stabilizer than Suc. Difficulties in maintaining active glycosidases in storage have previously been reported (e.g. Ross et al., 1993 and refs. therein).

α-Af I and II were present during fruit development (Fig. 4). α-Af I, a Zn-dependent enzyme (see

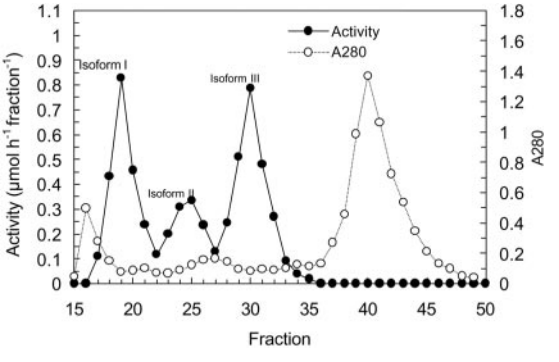

Figure 1. Separation of three α-Afs from ripe tomatoes using a Sephacryl S-200 column. Elution standards include: fraction 17, thyroglobulin from bovine thyroid (669 kD); fraction 19, bovine γ-globulin (158 kD); fraction 24, bovine serum albumin (BSA; 67 kD); fraction 25, ovalbumin from hen egg (43 kD); fraction 27, carbonic anhydrase (29 kD); fraction 30, cytochrome *c* from bovine heart (12.33 kD); and fraction 42, cyanocobalamin (1.35 kD). Elution was at pH 5 (see "Materials and Methods").

Table I. *Partial purification of three α-Afs from red-ripe tomato cv Jackpot fruit*

Protein extracts were subjected to SEC on Sephacryl S-200 and were size separated. α-Af isoforms were then bound to a column of concanavalin A-agarose and eluted with MM (see "Materials and Methods").

	α-Af	Protein	Activity	Specific Activity
		mg	*nmol min^{-1}*	*nmol min^{-1} mg^{-1}*
Crude extract	Total	100.0	253.0	2.5
Sephacryl S-200				
	Isoform I	5.9	76	12.9
	Isoform II	5.7	31	5.4
	IsoformIII	6.9	105	15.2
Concanavalin A				
	Isoform I	1.3	30	23.1
	Isoform II	0.8	9	11.2
	IsoformIII	0.9	40	44.4

below), was found at a relatively constant activity level (fresh weight basis) during the cell expansion phase, until maximal fruit size was reached at 35 to 40 DAA. Total α-Af I activity increased steadily in the fruit, keeping pace with fruit enlargement. Thus, α-Af I could be associated with the structural modification of cell walls that occurs during fruit growth. In contrast, α-Af II is highly active during early growth (Fig. 4). The activity of this isoform accounts for 80% of the total α-Af activity in 10-d-old fruits, but it declines during fruit ripening (Fig. 5).

α-Af III is barely detectable in MG 4 tomatoes (48 DAA), but activity increases with the onset of ripening (Fig. 5), reportedly in close correlation with the decrease in fruit firmness of cv VF36 (Sozzi et al., 1998). The highest α-Af III activity—as well as maximum softening—is found in red-ripe fruit (56 DAA). α-Af III activity is substantially lower in ESS fruit

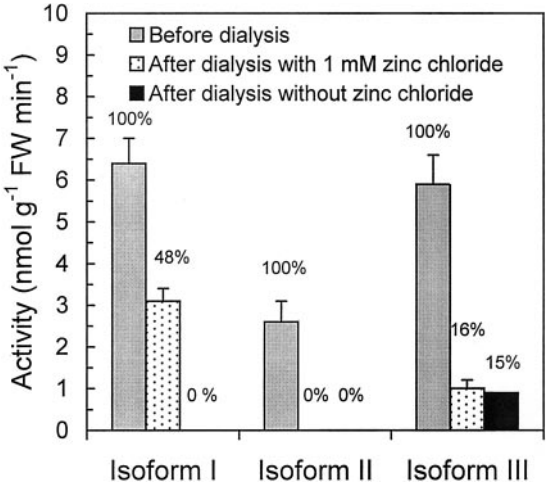

Figure 3. Effect of dialysis in the presence or absence of Zn^{2+} on α-Af isoforms. An aliquot of the concentrated crude extract from red ripe tomatoes was chromatographed on the Sephacryl S-200 column, and activity was determined and recorded on a fresh weight basis. Fractions corresponding to each peak were pooled and dialyzed against 100 mM sodium acetate, pH 5, with or without 1 mM $ZnCl_2$. Percentages indicate recovery of activity.

than in wild-type tomato pericarp and increases only slightly during ripening. However, ethylene treatment of these fruit promotes ripening, including fruit softening, and causes a significant increase in α-Af III activity, reaching a level similar to that measured in wild-type fruit.

All previous reports have addressed total α-Af activity in tomato fruits, ignoring the possibility that distinct isoenzymes contribute to the composite, total activity measured. The present study clearly demonstrates that a total α-Af activity analysis masks the isoform changes that are crucial to understanding

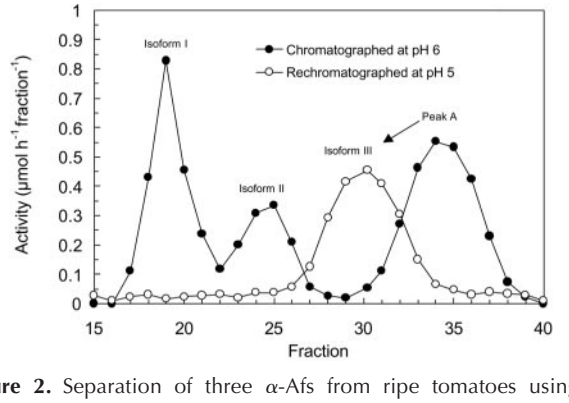

Figure 2. Separation of three α-Afs from ripe tomatoes using a Sephacryl S-200 column eluted at pH 6 (compare with elution pattern shown in Fig. 1). The fractions identified as "Peak A" were pooled, and the pH was adjusted to 5 with 0.5 M HCl. The pooled fractions were concentrated 10-fold with dialysis tubing placed against polyethylene glycol compound (PEGc). The concentrated extract was loaded on the Sephacryl S-200 column and was rechromatographed at pH 5.

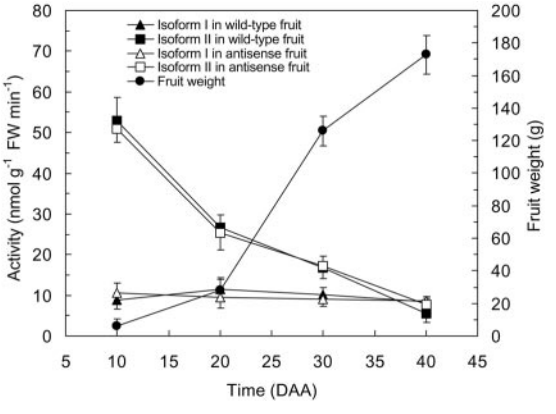

Figure 4. Activities of α-Af I and II during fruit growth. The activity of each isoform was calculated by measuring the area under the curve describing the assayed activities of the Sephacryl-S 200 column-fractionated isoforms. Days after anthesis (DAA) was calculated based on sampling of fruit developing from blossoms that were tagged upon opening. Data are means ± SD of three composite samples. Isoform III was not detected.

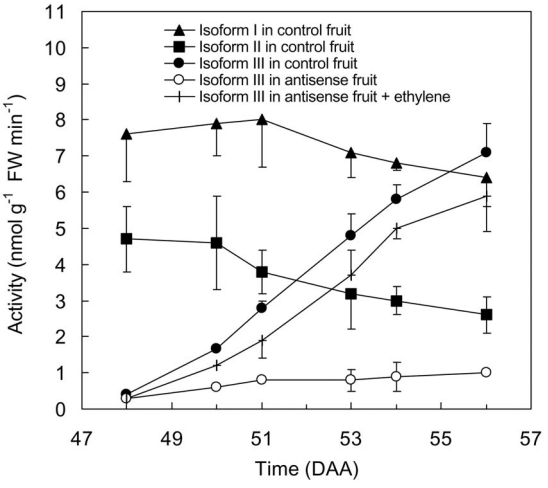

Figure 5. Activities of α-Af isoforms during fruit ripening. The activity of each isoform was calculated as described in the legend to Figure 4. Data are means ± SD of three composite samples. Activities of isoforms I and II were statistically not different in wild-type and ESS tomato fruit (data not shown). Ripening stages in control fruit are as follows: mature-green (MG 4; 48 DAA), breaker (50 DAA), turning (51 DAA), pink (53 DAA), light red (54 DAA), and red ripe (56 DAA).

α-Af roles. Cell wall modification during cell division, cell expansion, and early fruit ripening may involve glycosidases (e.g. β-Gal and α-Afs) acting with pectolytic enzymes such as polygalacturonase in pectin polymer metabolism (Hadfield and Bennett, 1998; Smith et al., 1998).

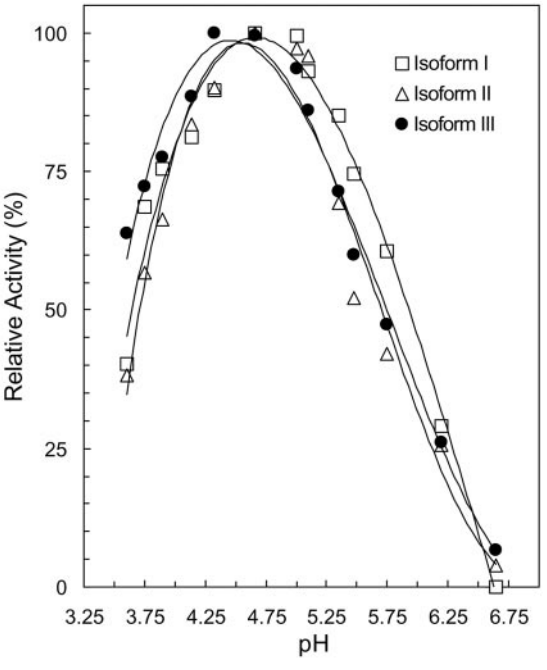

Figure 6. Effect of pH on the activities of α-Af I, II, and III from red ripe tomato. The buffers used were 0.2 M citric acid-sodium citrate (pH range 3.6–5.1) and 0.2 M KH_2PO_4-Na_2HPO_4 (pH range 5.1–6.7). When the reaction mixture was prepared with McIlvaine's buffer (pH range 3.6–6.7), no significant differences were detected.

α-Af I Is a Zn-Dependent Enzyme

α-Af isoforms hydrolyzed *p*-nitrophenyl-α-L-arabinofuranoside (*p*-NAf) well into the acid range with a pH optimum at 4.25 to 4.75 when tested with citrate and phosphate buffers (Fig. 6). The activities were not affected by the nature of the buffer. Screening experiments with metal ions were performed in the presence of NaCl and Suc (stabilizers, see below). The cations and the substrate were added to the reaction mixture simultaneously. These experiments showed that Hg^{2+} and Cu^{2+} hastened inactivation. Hg^{2+} (1 mM, final concentration) in the assay inhibited α-Af isoforms I, II, and III 52%, 64%, and 58%, respectively, and Cu^{2+} (1 mM) inhibited the three isoforms 28%, 14%, and 29%, respectively, suggesting the presence of sulfhydryl groups, or His and Trp residues. With the exception of Zn^{2+} (see below), the effects, if any, of other cations tested (5 mM Ca^{2+} or Mg^{2+}; 1 mM Mn^{2+}, Fe^{2+}, Ni^{2+}, Li^{2+}, or Co^{2+}) were too small (less than 10%) to have unequivocal significance (data not shown).

Isoform II and III activities were not affected by Zn^{2+} in a range of 0.3 to 5 mM, but the activity of α-Af I was strongly enhanced by the presence of this cation. This enhancement was identical when using chloride and sulfate salts. To gain insight into the function of this metal, pericarp tissue was homogenized and the enzyme was extracted in the presence and absence of 1 mM $ZnCl_2$. Aliquots of crude extract were partially purified using Sephacryl S-200 SEC and elution with 0.1 M sodium acetate, pH 5, ±1 M NaCl, 1% (w/v) Suc, and 1 mM $ZnCl_2$. The fractions corresponding to α-Af I were assayed in the presence or absence of $ZnCl_2$ (Fig. 7; Table II). Maximum ac-

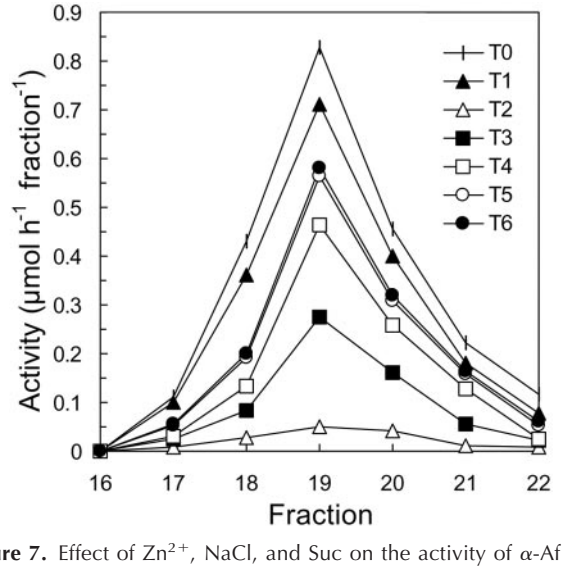

Figure 7. Effect of Zn^{2+}, NaCl, and Suc on the activity of α-Af I. A single sample of red ripe pericarp tissue was homogenized in a Waring blender to provide a uniform source of enzyme for testing. Sample handling, analysis, and assay are described in "Materials and Methods" and in Table II. Identical results were obtained when 30-d-old immature green fruit pericarp was used.

Table II. *Variations in protein extraction protocol, SEC conditions, and activity assays used in tests of α-Af I stability (see Fig. 7)*

Sample Name	Protein Extraction Buffer (as in "Materials and Methods," ± 1 mM $ZnCl_2$)	SEC Elution Buffer (100 mM acetate, pH 5, ± NaCl, Suc, and/or $ZnCl_2$)	Zn^{2+} in Assay
T0	+	1 M NaCl, 1% (w/v) Suc, and 1 mM $ZnCl_2$	None
T1	+	1 M NaCl and 1% (w/v) Suc	None
T2	−	No additions	None
T3	−	No additions	5 mM
T4	−	No additions	10 mM
T5	−	No additions	15 mM
T6	−	No additions	25 mM

tivity was observed when Zn^{2+} was used in the extraction and purification procedures, in the presence of NaCl and Suc. Even when the cation was not added during the purification procedure, most of the activity was conserved when NaCl and Suc were present. However, an almost total loss of activity was observed when the fractions were obtained and assayed in the absence of Zn^{2+}, NaCl, and Suc. A dose-dependent partial reversal of this loss was obtained when Zn^{2+} was added to the reaction mixture.

To further the study of this Zn-dependent enzyme, the active fractions obtained in the absence of Zn^{2+}, NaCl, and Suc were pooled and concentrated by dialysis against high-M_r PEGc. This concentrated extract was used to test the effect of 1,10-phenanthroline, a high-affinity Zn-chelating agent (Fig. 8). The addition of 1,10-phenanthroline inhibited the α-Af I activity in all cases, although this effect was significantly enhanced when the enzyme was preincubated with the chelator for 1 h prior to the addition of the substrate. This indicates that the presence of the substrate may have a protective effect on the active site.

For an element to be proven essential for an enzyme, it should be demonstrated that the enzyme cannot display catalytic activity in the absence of the element and that no other element can substitute for the test element. Zn^{2+} was the only cation found to restore the activity of α-Af I in decayed preparations (Fig. 9). Also, dialysis of the isoform was possible at pH 5 with reduced loss in activity when Zn^{2+} was present (Fig. 3).

Zn^{2+} is a cation with various coordination possibilities and several potential geometries. Thus, it is easily adaptable for different ligands and may play a catalytic role, a structural role, or both (Fox and Guerinot, 1998). This raises the question of the Zn content of α-Af I and its in vivo contribution to the enzyme properties. The presence of Zn^{2+} in the in-

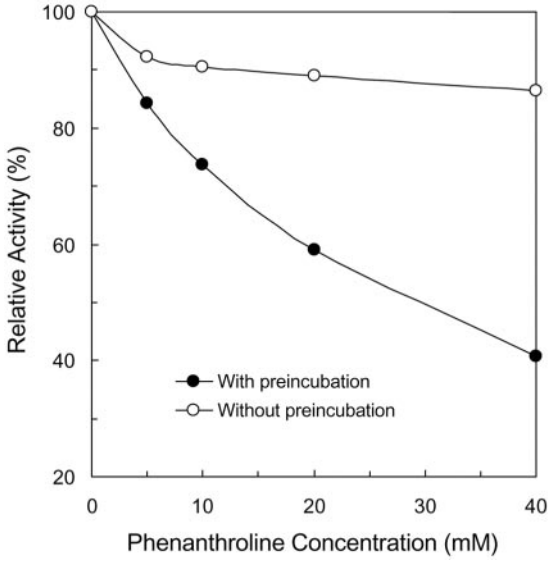

Figure 8. Effect of 1,10-phenanthroline on the activity of α-Af I. α-Af I was partially purified in the absence of Zn^{2+} or other stabilizers and concentrated. The enzyme was preincubated with different concentrations of the chelator for 1 h prior to the addition of the substrate, or the chelator and the substrate were added simultaneously. Results are expressed as percentages of the enzyme activity measured in the absence of the Zn-chelating agent.

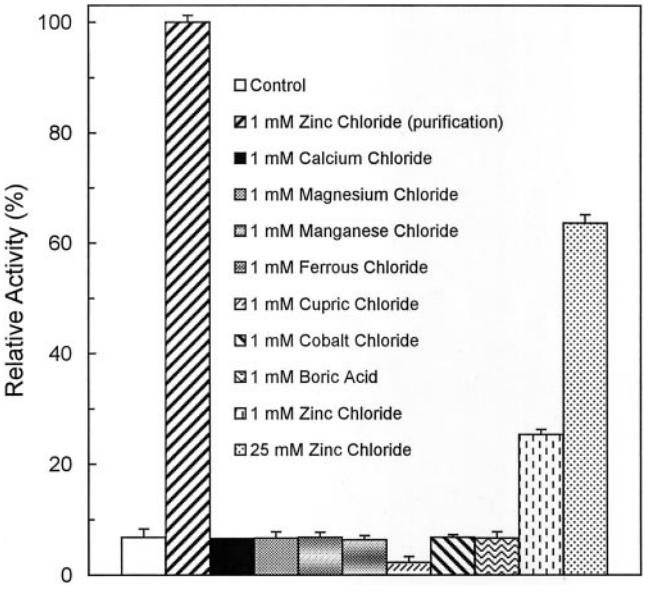

Figure 9. Effect of different metals on the activity of decayed preparations of α-Af I. The isoform was partially purified in the absence of Zn^{2+} and was concentrated 20-fold. The addition of the substrate and the cations to the reaction mixture was accomplished simultaneously.

cubation mixtures strongly improved the activity, and the addition of 1,10-phenanthroline had only a limited effect on activity in the presence of the substrate, suggesting that the catalytic site contains at least one Zn atom that is absolutely necessary, but not easily chelatable, when substrate is bound. The specific role played by Zn^{2+} in maintaining α-Af I activity in extracts and assays is not clear. However, the main role of structural Zn^{2+} in proteins is to stabilize tertiary structures. NaCl and Suc stabilize the enzyme even in the absence of Zn^{2+}, and are additives that may simultaneously change solvent structure and prevent conformational changes and the unfolding of proteins even under high temperature conditions (Gray, 1988; Devi and Rao, 1998). Thus, the ion's primary role in α-Af I may be in stabilizing its structure, perhaps in its active center.

Most microbial α-Afs are not altered by the presence of Zn^{2+} (Saha, 2000 and refs. therein). In contrast, this cation stimulates the activity of the α-Afs purified from carrot (*Daucus carota*) cells (Konno et al., 1987) and soybean (*Glycine max*) cotyledons (Hatanaka et al., 1991). Zn was found to prevent the loss of enzymatic activity of α-mannosidases (Snaith and Levy, 1968).

Experiments with α-Af I provide evidence of the involvement of Zn^{2+} in tomato cell wall depolymerization/turnover. Different cell wall fractions, such as pectins and xyloglucans, contain measurable traces of Zn (Fry, 1998). Thus, Zn could be playing structural and mechanistic roles in fruit cell walls. Zn deficiency occurs in the western United States, in general, and on irrigated lands in California, in particular (Swietlik, 1999). This may explain the changes in α-Af I activity levels measured, in the absence of added Zn^{2+}, in different commercial batches of the same tomato cultivar sampled at the same ripeness stage (data not shown).

Activity against Native Substrates

Free Ara was detected after the incubation of α-Af I with the 1 M KOH- and 4 M KOH-soluble fractions (KOH-Fs) of MG tomato cell walls, and after the incubation of α-Af II and III with the Na_2CO_3-soluble fraction (Na_2CO_3-F; Table III).

Two types of arabinosidase action are known (Dey and del Campillo, 1984): arabinopyranosidase and arabinofuranosidase activity. The first, unlike the arabinofuranosidase activity, is relatively unspecific and is also exhibited by different galactosidases. This is attributable to the similar ring structures of arabinopyranosides and galactopyranosides and the lack of specificity of galactosidases for functional groups at C-6 of their substrates. Very few arabinosyl residues adopt a six-membered ring configuration in the cell wall; the L-Ara units generally assume the α-furanose conformation in most Ara-containing polysaccharides: arabinans, arabinogalactans, arabinoxyloglucans, and glucuronoarabinoxylans (Carpita and Gibeaut, 1993; Pérez et al., 2000). However, the α-pyranose conformation is found in some pectic polysaccharides (Huisman et al., 2001). Some degree of synergy in substrate disassembly could have occurred during incubation because of the activity of other glycosidases in the incompletely purified α-Af enzyme preparations; activities that might be required for concerted action in the breakdown of the natural substrates and arabinosidase release of monosaccharide Ara (e.g. de Vries et al., 2000).

Cell wall Ara content decreases steadily during ripening of whole tomato fruit (Gross and Wallner, 1979) and explanted pericarp discs (Campbell et al., 1990). Polysaccharides in the W-F were not a target for any α-Af tested (Table III). Ara residue content increases 3- to 4-fold in the W-F polymers during ripening (Carrington et al., 1993), probably due to the accumulation of polysaccharides solubilized in vivo, but still relatively intact and loosely associated with the cell wall. Also, there was no apparent release of Ara from the CDTA-F when incubated with the α-Afs (Table III). Carrington et al. (1993) reported that a decrease in Gal, but not in Ara, takes place in vivo in this fraction during ripening. In contrast, we detected a release of Ara from the pectic $NaCO_3$-F when incubated with the α-Af II and III extracts. Thus, these isoforms could be responsible for the observed decrease of Ara content in the Na_2CO_3-F in wild-type fruit during ripening (Carrington et al., 1993). The α-Af-catalyzed release of arabinosyl residues from this substrate was higher when incubated with the isoform II than isoform III, although the activity in the α-Af III extract used was capable of releasing *p*-nitrophenol from the artificial *p*-NAf at a 5-fold

Table III. *Release of Ara by partially purified α-Af I, II, and III acting on a range of MG tomato fruit cell wall fractions*

The activity against the different cell wall fractions was assayed as detailed in "Materials and Methods." Data are expressed as the percentages of the arabinosyl residues in each of the cell wall fractions used as substrates that were released as free (i.e. monosaccharide) Ara during incubation. Differing amounts of arabinosyl residues were presented with the extracted substrates. The Na_2CO_3-F contained approximately 2,100 μg of Ara, the 1 M KOH-F contained approximately 880 μg, and the 4 M KOH-F contained approximately 1,000 μg. The water soluble fraction (W-F) and cyclohexane diamine tetraacetic acid-soluble fraction (CDTA-F) substrates used for the incubations each contained approximately 1,000 μg of arabinosyl residues. The activities of the α-Af isoforms used in incubations, as determined in assays with *p*-NAf, were 3.1, 6.2, and 30.2 nmol *p*-nitrophenol min^{-1} for α-Afs I, II, and III, respectively. No free Ara was detected in any of the substrates or enzyme blanks.

α-Af	Cell Wall Fraction				
	W-F	CDTA-F	Na_2CO_3-F	1 M KOH-F	4 M KOH-F
Isoform I	–	–	–	3.3%	1.6%
Isoform II	–	–	0.4%	–	–
Isoform III	–	–	0.2%	–	–

–, No free Ara detected.

higher rate than did the amount of α-Af II used. These results could be artifactual, a consequence of differential "access" of the enzymes to the simple, synthetic glycoside substrate and the extracted polymers as they are presented in solution. The availability of the wall-localized enzyme targets will certainly be affected by several factors not present in vitro. However, these results may indicate that α-Af II and III are isoforms with different substrate specificities, targeting distinct linkages within the same fraction.

The hemicelluloses are not extracted from the cell walls by water or by solutions of chelating agents, but can be extracted by relatively strong alkali solutions, typically 1 to 4 M KOH. This probably applies to glucuronoarabinoxylans and arabinoxyloglucans, which may be strongly hydrogen bound to cellulose microfibrils. Our results suggest that α-Af I may target one of those cell wall polymers (Table III). Because the arabinoxyloglucans accumulate during cell elongation and become a major cellulose crosslinking polysaccharide, the hydrolysis of these glycans may be necessary for wall expansion during fruit growth.

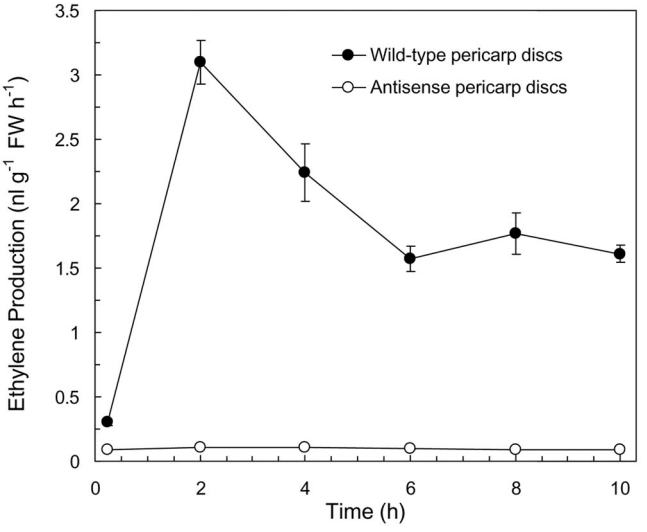

Figure 10. Ethylene biosynthesis following excision of control and ESS tomato pericarp discs. Pericarp discs were prepared from a 2-cm band at the equatorial regions of three fruits. Values are an average for 12 discs ± SD. Where bars are not shown, the SD did not exceed the size of the symbol.

General Features of the Antisense ACC Synthase Tomato Pericarp Disc System

ACC synthase is the rate-limiting enzyme in the ethylene synthesis pathway (Yang and Hoffman, 1984). *LE-ACS2* and *LE-ACS4* are the two genes encoding ACC synthase that are expressed during tomato fruit ripening. The expression of antisense *LE-ACS2* RNA inhibits the expression of both genes, *LE-ACS2* and *LE-ACS4* (Oeller et al., 1991). Thus, ESS fruit display low ethylene biosynthesis (less than 0.5% of normal levels). *LE-ACS2* and *LE-ACS4* are also responsible for wound-induced ethylene production (Lincoln et al., 1993). In addition, *LE-ACS3*, a major transcript in cell cultures, is induced after wounding, although it does not accumulate in ripening intact tomatoes (Yip et al., 1992). For this study, ESS tomato pericarp discs were checked to provide data on the degree of inhibition of excision-stress ethylene biosynthesis.

Wild-type fruit displayed a transient, sharp increase in ethylene production right after disc excision (Fig. 10), as previously described (Campbell et al., 1990). Stress ethylene rose 10-fold, from 0.3 nL g^{-1} fresh weight h^{-1} (15 min after excision) to 3.1 nL (2 h after excision). In contrast, wound-induced ethylene production in ESS discs was reduced >98% during the first 10 h after excision, relative to that of discs from control fruit (Fig. 10). Furthermore, there were no changes in the rate of ethylene biosynthesis in these discs during the 9-d experimental period (data not shown).

The negligible biological impact of wound-induced ethylene biosynthesis in ESS discs was confirmed indirectly by means of color measurements. ESS pericarp discs changed their color in a pattern similar to that of intact ESS fruit (Fig. 11). There was minimum degradation of chlorophyll and no accumulation of lycopene, according to a* values. When continuously treated with 15 μL L^{-1} ethylene, the development of red color by ESS discs was statistically the same as that of ripening control fruit discs (Fig. 11). Elimination of ACC synthase gene expression results in discs that ripen only if they are treated with ethylene, and concentrations of exogenous ethylene can be readily manipulated, thus simulating levels similar to those in wild-type ripening fruit. Therefore, ESS tomato pericarp discs provide a simple and promising system for studying the biochemical and physiological responses of mature fruit tissue to different metabolic regulators. This system can be used to evidence or eliminate interactions between ethylene and other hormonal signals, to dissect signal transduction pathways, and to perform studies involving the addition of labeled tracers (e.g. Greve and Labavitch, 1991) without confusion arising from wound ethylene presence.

α-Af Isoforms Display Differential Responses to Auxin Analogs, Gibberellic Acid (GA_3), and Ethylene

Pericarp discs from 48-d-old (MG 4) ESS fruit were treated with various plant growth regulators. Activities of the α-Af isoforms were followed by extracting proteins from the discs and chromatographing them on Sephacryl S-200. Treatments with GA_3 and the auxin analogs 2,4-dichlorophenoxyacetic acid (2, 4-D) and α-naphthaleneacetic acid (NAA) caused increases in α-Af I activity (Fig. 12A). The effects of the growth regulators were quite different for α-Af II.

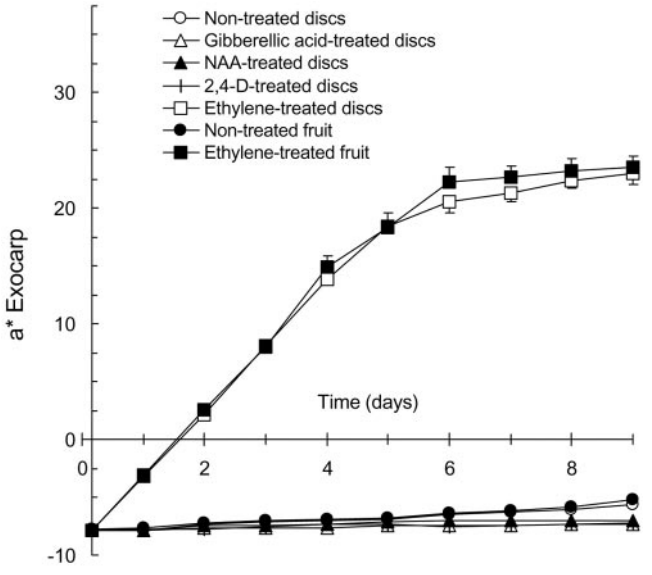

Figure 11. Changes in color of exocarp tissue during ripening of discs cut from ESS fruit in response to plant growth regulators. Intact and excised fruits were obtained from the first truss of two plants. Disc color is an average of 24 discs (two fruits) measured through the plastic bottom of the storage plate and mathematically converted to direct color measurements. Ethylene-treated intact fruit color is an average of 24 equatorial readings (two fruits). Where bars are not shown, the SD did not exceed the size of the symbol. Green pericarp tissue has a* values in the range from −15 to −5, turning tissue has values in the range from 0 to 5, and red ripe tissue has values >15.

GA_3 promoted α-Af II activity. Three days after treatment, the activity in the control discs had decreased 20%, whereas the activity of α-Af II almost doubled in the GA_3-treated discs. Activity in GA_3-treated discs remained 2- to 3-fold higher than that of controls for the 9-d post-treatment period (Fig. 12B). GA_3 treatment of MG control tomato pericarp discs delays ripening, perhaps because it reduces the normal increase in ethylene synthesis (Ben-Arie et al., 1995). Therefore, the decrease in α-Af II activity normally seen in ripening tomatoes may be due to the loss of a gibberellin-like factor responsible for maintenance of α-Af II activity. The fact that this α-Af activity fell in control and ethylene-treated ESS discs indicates that ethylene has little to do with the normal ripening-related decrease in α-Af II activity. NAA had no effect on α-Af II, whereas 2,4-D had an impact only after 6 d. This effect was smaller than that of GA_3. Synthetic auxins like 2,4-D and NAA have been extensively employed as indole-3-acetic acid substitutes because of their relative stability against peroxidative activity, which is known to degrade indole-3-acetic acid (Kokkinakis and Brooks, 1979). Nevertheless, fruit tissue response differed according to the synthetic auxin applied. 2,4-D has been shown to promote α-Af synthesis during normal growth of carrot cell cultures (Konno et al., 1999).

α-Af III proved to be the only isoform directly influenced by ethylene (Fig. 12C). The continuous ethylene treatment enhanced extractable activity 5-fold. Moreover, the concentration of ethylene in ESS discs (under 0.1 nL g^{-1}), although insufficient to induce some biosynthetic processes (i.e. lycopene production), was sufficient to cause a slight increase in disc α-Af III activity after 3 d. The same low ethylene concentration induced a minor increase in the β-Gal II activity of intact fruit (Sozzi et al., 1998). Polygalacturonase mRNA and protein accumulations have proven to be responsive to low concentrations of ethylene (Sitrit and Bennett, 1998). ESS tomatoes exhibit evidence of a delay in senescence, and most of

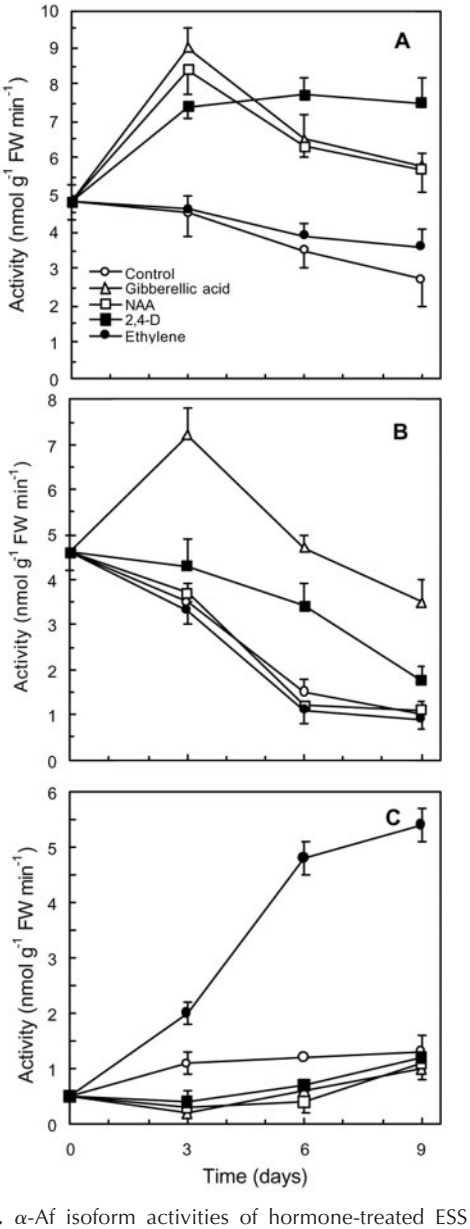

Figure 12. α-Af isoform activities of hormone-treated ESS tomato pericarp discs. A, Isoform I; B, isoform II; C, isoform III. The activity of each isoform was calculated as described in the legend to Figure 4. Values represent the means ± SD of two (d 3 and 6) or three (d 0 and 9) 24-disc composite samples. Where bars are not shown, the SD does not exceed the size of the symbol.

the ultrastructural organization is not affected over time. Nevertheless, some softening does take place, and less electron-dense areas in the middle lamella have been detected (Sozzi et al., 2001), which may indicate some action of pectolytic or other wall-degrading enzyme activities.

α-Af III activity was transiently reduced in untreated discs when GA_3, 2,4-D, and NAA were applied (Fig. 12C). Ethylene is directly involved in tomato fruit ripening, but it is likely that the triggering of ripening is due to the influence of a number of hormonal factors. The decline of auxins and gibberellins prior to the ethylene burst may be part of that control process. Our results demonstrate that physiological levels of auxins and GA_3 antagonize the effect of low ethylene concentrations on the ripening-related α-Af III. Ben-Arie et al. (1996) have shown that preharvest GA_3 treatment of persimmon (*Diospyros kaki*), another climacteric fruit, delays the loss of cell wall arabinosyl residues during ripening.

Although it is clear that none of the results shown herein can be ascribed to wounding, the possibility that α-Af isoforms may be regulated in ways not described here should not be ruled out. Nevertheless, their patterns of change in intact fruit are consistent with those measured in hormone-treated antisense discs. These results strongly suggest that tomato fruit α-Afs are a divergent family of enzymes of at least three members, as inferred from their different activity profiles during fruit ontogeny, their properties (e.g. different elution points from Sephacryl S-200, α-Af I is the only Zn-dependent isoform), their different activities against cell wall fractions, and the hormonal influences described in Figure 12. Thus, tomato α-Afs are likely to be encoded by a gene family, as found for all other cell wall-modifying enzymes to date (Brummell and Harpster, 2001), because the release of neutral sugars from the cell wall starts before the climacteric ethylene rise. If α-Af isoenzymes attack different in vivo targets, that could position isoform III as a candidate for a softening-related depolymerizing activity, even when there is Ara release from the cell walls during growth, a phase in which no softening takes place. The overlapping activities of these arabinosidases during ripening also suggests a specificity of function on different substrates or cell wall microstructural domains: α-Afs I and II could promote discrete modifications of cell wall architecture during growth and expansion, but α-Af III may be involved in the major cell wall breakdown that takes place during ripening.

MATERIALS AND METHODS

Plant Material and Chemicals

For partial purification and characterization of α-Afs, red-ripe tomato (*Lycopersicon esculentum* cv Jackpot) fruits were obtained from a commercial farm near Davis, California. For the preparation of different cell wall fractions, MG fruit were collected from greenhouse-grown plants (cv Castlemart). For experiments testing the effect of ethylene on tomato fruit α-Afs and for tissue-culture experiments, we used transgenic seeds expressing an antisense ACC synthase RNA in the background line VF36 (A11.1, designated ESS). Forty plants of each type were grown under daylight in 15-liter plastic pots in a greenhouse at the University of California (Davis). Cultural practices were performed as previously described (Sozzi et al., 1998). Three flowers per inflorescence were tagged at anthesis and the others were removed. Experiments were performed with fruits from the first truss. Control and ESS fruit were harvested at the reported DAA. Control and antisense fruit (48-d-old, staged as MG 4, all locules liquefied, slight pinkish color to locules but no exterior pink color) were harvested and stored at 20°C ± 1°C in humidified air and diffuse light until used. A sample of 48-d-old ESS fruit was enclosed in 4-L glass jars (two tomatoes per jar) and was exposed to a mixture of 100 ± 0.5 μL L^{-1} ethylene in humidified air using a constant flow-through gas system. The desired ethylene concentration was reached within 1 h after placing the fruit into the containers. Flow rates (1,100 mL min^{-1}) selected ensured that CO_2 accumulation would not exceed 0.2%. CO_2 accumulation was checked once a day using an infrared CO_2 analyzer (model PIR-2000R; Horiba Instruments, Irvine, CA). All chemicals were from Sigma Chemical (St. Louis).

α-Af Extraction, Isoform Separation, and Partial Purification

Composite pericarp samples were homogenized in a Waring blender (45 s) and a Polytron (45 s) with 3 volumes of 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) polyvinylpolypyrrolidone. The suspension was stirred for 30 min, centrifuged at $12 \times 10^3 g$ for 15 min, and filtered through glass fiber filter paper (GF/C; Whatman, Clifton, NJ).

The filtrate was concentrated 8- to 12-fold with regenerated cellulose dialysis tubing (Spectra/Por 1, 6,000–8,000 M_r cut off; Spectrum Laboratories, Rancho Dominguez, CA) placed against PEGc (M_r 15,000–20,000). An aliquot of the concentrated extract was loaded onto a Sephacryl S-200 column (35 × 2.6 cm) previously equilibrated with 100 mM sodium acetate, pH 5, containing 1 M NaCl and 1% (w/v) Suc. The column was eluted with the same buffer, and 3.3-mL fractions were collected at a flow rate of 2 mL min^{-1}. To quantify the activity of each isoform, fractions containing overlapped activities from two α-Afs were concentrated and rechromatographed. Activities of separated α-Af isoforms in fruits and discs were calculated by summing the activities identified in the fractions corresponding to each Sephacryl-S 200 column-fractionated peak.

Fractions from each peak of activity were pooled, pH was adjusted to 6 using 0.5 M NaOH, and 1 mM of each $CaCl_2$, $MnCl_2$, and $MgCl_2$ was added. Further enrichment of each peak was achieved using a 5-mL concanavalin A (immobilized on 4% [w/v] beaded agarose) column, which had been equilibrated with 100 mM sodium acetate, pH 6, containing 1 M NaCl and 1 mM each of $CaCl_2$, $MnCl_2$, and $MgCl_2$. After loading, the column was washed with five column volumes of equilibration buffer. Bound α-Afs were eluted with five column volumes of 0.75 M MM in equilibration buffer. The MM was separated from the active fractions using a Sephadex G-25 minicolumn equilibrated and eluted with 100 mM sodium acetate, pH 4.5, containing 1 M NaCl. Unless stated otherwise, peak fractions from the last chromatographic step, containing α-Af I, II, and III activities, were used for enzyme characterization studies and assays against native substrates.

Protein Measurement and Enzyme Assay

Protein concentration was assayed by the Coomassie Blue G dye-binding method using the Bio-Rad reagent (Bio-Rad, Richmond, CA) and BSA as standard. Also, A_{280} was used to estimate protein concentration after the Sephacryl S-200 chromatographic step.

α-Af activity was measured using *p*-NAf as substrate. Unless otherwise indicated, the reaction mixture consisted of 250 μL of 0.1 M citrate buffer, pH 4.5, 200 μL of 0.1% (w/v) BSA, 50 μL of enzyme solution (or an appropriate dilution), and 200 μL of 13 mM substrate solution. After 1 h at 37°C, the reaction was stopped by addition of 1 mL of 0.13 M Na_2CO_3. Blanks (time 0) were prepared by adding Na_2CO_3 prior to the addition of substrate. Absorbance was measured at 400 nm. Free *p*-nitrophenol was used as standard. Enzyme activity calculations were based on enzyme dilutions that gave a linear increase in free *p*-nitrophenol over the course of a 3-h incubation.

Extraction of Cell Wall Fractions and Activity against Native Substrates

Pericarp tissue from MG tomato fruit (cv Castlemart) was sliced and immediately dropped into 3 volumes of 80% (w/v) ethanol and homogenized (Waring blender and Polytron). The homogenate was boiled for 30 min, allowed to cool, and filtered through glass filter paper. The retentate was washed with 95% (w/v) ethanol extensively. The solids were then resuspended in 3 volumes of chloroform:methanol (1:1), stirred for 15 min, and filtered. The retentate was washed with an additional 2 volumes of the same solvent. Insoluble material was washed with acetone until it was decolorized, and was then air-dried in a hood and in a vacuum oven overnight.

Cell wall fractionation was performed as previously described (Carrington et al., 1993), with minor differences. Briefly, 3 g of cell wall material was stirred overnight at room temperature with 300 mL of water containing 0.02% (w/v) Thimerosal and was filtered. The supernatant, designated W-F, was removed. Sequential extraction of the pellet with 0.05 M CDTA in 0.05 M NaOAc, pH 6, containing 0.02% (w/v) Thimerosal (overnight), 0.1 M Na_2CO_3 in 20 mM $NaBH_4$ (overnight), 1 M KOH in 20 mM $NaBH_4$ (4 h), and 4 M KOH in 20 mM $NaBH_4$ (4 h), produced the CDTA-F, Na_2CO_3-F, and 1 M KOH-F and 4 M KOH-F. pH was adjusted to 5 with glacial CH_3COOH in the case of the 1 M KOH-F and 4 M KOH-F. All the fractions were dialyzed exhaustively for 2 d at 4°C against several changes of 0.05 M sodium acetate, pH 4.5, and the volume of each dialyzate was recorded. Aliquots of each fraction were freeze-dried and hydrolyzed with 2 N trifluoroacetic acid for 1 h at 121°C. The resulting monosaccharides were reduced to alditols using $NaBH_4$ and were converted to alditol acetates (Blakeney et al., 1983), which were analyzed by gas chromatography (Campbell et al., 1990). After determining the level of cell wall-associated Ara in each fraction, the fractions were concentrated using regenerated cellulose dialysis membrane (M_r cutoff of 6,000–8,000) and PEGc to ensure that a potential enzymatic release of 0.1% of the total Ara would be detectable.

The reaction mixture contained 2 mL of substrate (pH 4.5), 1 mL of an α-Af isoform in 0.1 M sodium acetate, pH 4.5, containing 1 M NaCl, and 20 μL of toluene acting as a bacteriostat. In the case of α-Af I, $ZnCl_2$ was added to a final concentration of 1 mM. The α-Af I, II, and III-containing extracts incubated with wall-derived substrates were capable of releasing 3.1, 6.2, and 30.2 nmol *p*-nitrophenol min^{-1} in the standard assay with *p*-NAf. After incubation with wall-derived substrates at 35°C for 24 h, 1 mL of ethanol 100% (v/v) was added to 100 μL of the reaction mixture to precipitate undegraded polysaccharide and protein. The supernatant was recovered after centrifugation and was evaporated to dryness under a stream of filtered air. The dried samples were converted to alditol acetates (above), but without the acid hydrolysis step, and were analyzed by gas chromatography-mass spectrometry (Greve and Labavitch, 1991) to determine whether monosaccharide Ara had been released.

Antisense ACC Synthase Pericarp Disc Preparation and Characterization

Prior to excision of pericarp discs, 48-d-old control and ESS tomato fruit were sterilized for 10 min in 1% (w/v) sodium hypochlorite, thoroughly rinsed in sterile deionized water (sdH_2O), and dried in a laminar flow hood, where all the subsequent operations were performed as described (Campbell et al., 1990). In brief, fruits were bisected, and pericarp discs, 13 mm in diameter, were excised under aseptic conditions with a cork borer from the equatorial region of the outer pericarp, between the junctures of the radial septa. Discs were then sliced by hand to a uniform thickness of 6 mm, briefly rinsed twice with sdH_2O, drained, and blotted, with the cut surface down, on sterile filter paper for 1 min. Discs from all fruit were combined, randomized (each plate contained four discs from each of the six fruits), and placed with the epidermis side down in sterile 24-well tissue culture plates (Falcon 3047; Becton-Dickinson, Lincoln Park, NJ). Spaces between wells were partially filled with sdH_2O.

Unbuffered solutions of 100 μM 2,4-D, 100 μM NAA, and 100 μM GA_3 were sterilized by filtration through 0.2-μm sterile acrodiscs (Gelman Sciences, Ann Arbor, MI). Aliquots of 10 μL were distributed by micropipette in several droplets across the cut surface of each disc within 30 min of each disc's preparation. Control discs were treated with 10 μL of sdH_2O. Plates were stored in a box flushed with water-saturated air under isothermal conditions (20°C) until used. Another set of plates containing discs treated with 10 μL of sdH_2O was flushed with 15 ± 0.3 μL L^{-1} ethylene in humidified air. A sample of whole fruits was treated with the same controlled atmosphere. Discs were removed from the plates only for determining α-Af isoform activity.

Ethylene production was measured by gas chromatography at 80°C with an alumina column and was quantified by the integration of the peak from a flame ionization detector (Saltveit and Yang, 1987). The procedure for obtaining the gas samples was described in detail by Campbell et al. (1990). Surface color measurements were conducted using a reflectance spectrophotometer (model CR-200; Minolta, Osaka). One 24-disc plate per treatment was used to measure the a* values (hue on a green [−] to red [+] axis), and through-plate measurements were converted to direct color measurements using the corresponding regression equation (Campbell et al., 1990). Color measurements were also obtained from the equatorial regions of intact fruit exposed to air and ethylene.

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