A New 1-Aminocyclopropane-1-Carboxylic Acid-Conjugating Activity in Tomato Fruit

Melinda Neal Martin1*, Jerry D. Cohen, and Robert A. Saftner
Horticultural Crops Quality Laboratory, Beltsville Agricultural Research Center, Agricultural Research Service, United States Department of Agriculture, Beltsville, Maryland 20705

A new conjugate, 1-(γ-L-glutamylamino)cyclopropane-1-carboxylic acid (GACC), of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) is identified. The only previously identified conjugate of ACC is 1-(malonylamino)cyclopropane-1-carboxylic acid (MACC). GACC, not MACC, was the major conjugate formed by crude protein extracts of tomato (Lycopersicon esculentum Mill cv Alisa Craig) fruit pericarp and seeds incubated with [14C]ACC. GACC was resolved from [14C]ACC and [14C]MACC by reversed-phase C18 thin-layer chromatography and subsequently detected and quantified using a radioisotope-imaging system. Proteins precipitated from crude extracts failed to catalyze formation of GACC unless the supernatant was added back. Reduced glutathione, but not other reducing agents, replaced the crude supernatant. When [35S-cysteine]glutathione and [3H-2-glycine]glutathione were used as substrates, neither radiolabeled glycine nor cysteine from the glutathione tripeptide was incorporated into GACC. Oxidized glutathione, S-substituted glutathione, and di- and tripeptides having an N-terminal γ-L-glutamic acid, but lacking cysteine and glycine, also served as substrates for GACC formation. Peptides lacking the N-terminal γ-L-glutamic acid did not serve as substrates. Acid hydrolysis of GACC yielded ACC, suggesting that GACC is an amide-linked conjugate of ACC. Taken together, these results indicate that GACC is 1-(γ-glutamylamino)cyclopropane-1-carboxylic acid and that its formation is catalyzed by a γ-glutamyltranspeptidase. Gas chromatography-mass spectrometry analysis of the N-acetyl dimethyl ester of GACC confirmed this structure.

Conjugation has long been recognized as an alternative to destructive catabolism for detoxifying, inactivating, or sequestering unwanted foreign compounds in many eukaryotic organisms, including plants (Casida and Lykken, 1969; Caldwell, 1986; James, 1986; Lamoureux and Rusness, 1986; Wilkinson, 1986). In plants, conjugated forms of hormones and messengers, including IAA, GAs, ABA, cytokinins, jasmonic acid, and salicylic acid are ubiquitous (Schreiber et al., 1986). For example, as many as 20 ester and amide conjugates of IAA have been identified (Slovin et al., 1994). The extent to which the conjugates of plant hormones, like those of foreign compounds, are sequestered or inactivated end products or represent reversible homeostatic systems for regulating the levels of free hormones has not been definitively established. In fact, the "active" form(s) of many hormones is unknown, as are the factors that regulate the levels of free versus conjugated forms of hormones.

The ethylene molecule is not a substrate for conjugation reactions, and its gaseous state and ready diffusion from plant tissues may reduce the need for conjugation. Until now, a single conjugate of the ethylene precursor ACC has been isolated and identified as MACC (Yang, 1986). MACC was formed following application of ACC to buckwheat hypocotyls (Amrhein et al., 1981) or wheat leaves (Hoffman et al., 1982). Likewise, an endogenous conjugate, isolated from peanut seed (Hoffman et al., 1983) and cocklebur seed (Satoh and Esashi, 1984), has been identified as MACC. ACC N-malonyltransferase, which catalyzes the malonyl-CoA-dependent formation of MACC from ACC, has been detected in tissue extracts from a number of sources, including grapefruit flavedo (Liu et al., 1985a), mung bean hypocotyls (Kionka and Amrhein, 1984), and tomato (Lycopersicon esculentum) fruit (Liu et al., 1985b), and has been purified from mung bean hypocotyls (Guo et al., 1992) and the pericarp of tomato fruit (Martin and Saftner, 1995). In many other cases, conjugation of ACC (presumably to MACC) has been reported, but identification was often based solely on co-chromatography with synthetic MACC or on ethylene formation following acid hydrolysis to ACC and subsequent chemical oxidation to ethylene as described by Lizada and Yang (1979).

Su et al. (1984) reported that in tomato fruit both ACC and an unidentified acid-hydrolysable conjugate of ACC increased during fruit development and that, like ethylene biosynthesis, both ACC and conjugated ACC increased dramatically with the onset of ripening. In this paper, we present results that show that, at least in tomato fruit, tissue extracts have the ability to conjugate ACC to another conjugate in addition to MACC. We describe the resolution of a new conjugate of ACC (GACC) from extracts of tomato fruit incubated with [14C]ACC, which, like MACC, is acid hydrolysable to ACC. We also describe the identification of the enzymatic activity catalyzing the formation of GACC and the developmental and spatial patterns of this activity in seeds and maternal tissues of tomato fruit.

Abbreviations: GACC, 1-(γ-L-glutamylamino)cyclopropane-1-carboxylic acid; GST, glutathione S-transferase; HPTLC, high-performance thin layer chromatography; MACC, 1-(malonylamino)cyclopropane-1-carboxylic acid.

1 Present address: Climate Stress Laboratory, Beltsville Agricultural Research Center, Agricultural Research Service, Beltsville, MD 20705.
2 Corresponding author; fax 1–301–504–7521.
MATERIALS AND METHODS

Plant Material

Fruit of tomato (*Lycopersicon esculentum* Mill cv Ailsa Craig) were used throughout. Plants were grown in a greenhouse in the winter and in a greenhouse or in field plots during the summer. Fruit were harvested at the stages indicated for each experiment and treated as described below.

Preparation of Tissue Extracts for Enzyme Assays

Locular fluid was removed from seeds by mixing at low speed in a Waring® blender or in a Wheaton (Millville, NJ) Potter-Elvehjem tissue grinder with a Teflon pestle. Neither separation process broke the seeds. Seeds and maternal tissue were frozen in liquid nitrogen and ground to a powder with a mortar and pestle for small-scale preparations. For large preparations, maternal tissues were frozen in liquid nitrogen and ground in a Waring blender, and seeds were powdered in a coffee grinder. The ground tissue was extracted with buffer A, which contained 100 mM Tris-Cl, pH 8.0, 2 mM EDTA, 5 mM DTT, 1 mM benzanilide, 1 mM 6-amino-n-hexanoic acid, 1 mM PMSF, 250 μM N-α-p-tosyl-l-arginine methyl ester, 250 μM N-tosyl-l-phenylalanine chloromethyl ketone, 3 μM pepstatin, and 1 μM leupeptin. Two milliliters of buffer per gram of maternal tissue and 5 mL of buffer per gram of seeds were used to prepare crude protein extracts.

Assays were performed as described below using the crude protein extracts directly or after centrifugation for 10 min at 10,000g to remove cell debris. Where indicated, crude protein extracts were precipitated at 5°C with ammonium sulfate (65% saturation), ethanol (70%, v/v), or acetone (50%, v/v). The precipitated proteins were recovered by centrifugation for 10 min at 10,000g, resuspended in buffer containing 100 mM Tris-Cl, pH 8.0, 2 mM EDTA, and other components of buffer A at one-fifth concentration, and dialyzed overnight against the same buffer. Recovery of GACC-forming activity was nearly 100% in each case. Much of the ACC N-malonyltransferase activity was lost following acetone or ethanol precipitation. Following precipitation of the protein, the ethanol or acetone in the supernatant was evaporated in vacuo at 30 to 35°C, and this supernatant was used for reconstitution experiments. Protein concentrations were determined as described by Bradford (1976) using a Bio-Rad kit and BSA as a standard.

Assay of ACC N-Malonyltransferase Activity

The assays contained in a volume of 50 μL: enzyme, 100 mM Tris-Cl, pH 8.0, 1 mM DTT, 1 mM EDTA, 2 mM malonyl-CoA, and 2 mM [2,3-14C]ACC (270 nCi/assay). [2,3-14C]ACC with a specific activity of 53.4 μCi/mmol was obtained from Research Products International (Mount Prospect, IL). The reactions were initiated with [14C]ACC, incubated for 15 to 120 min at 30°C, and terminated by the addition of 50 μL of absolute ethanol. Usually, 5 μL of each assay were spotted onto a lane of an Analtech (Newark, DE) HPTLC-GHILF normal-phase silica gel TLC plate (10 × 20 cm) and rapidly separated using a solvent system of 1-propanol:ammonia (6:4, v/v) to resolve [14C]ACC and [14C]MACC. Prior to chromatography, assays containing crude protein extracts were centrifuged in the same microcentrifuge tubes in which the assays were performed for 5 min at 13,000g to pellet protein and other debris. When crude protein extracts were used in the assays, chromatography was performed on J.T. Baker reversed-phase octadecyl (C18) TLC plates in a solvent system of 1-propanol:water (8:2, v/v). Two conjugates, [14C]MACC and [14C]GACC, were formed in crude extracts and were resolved only when the reversed-phase system was used. Reversed-phase TLC was used only when necessary, since cost was greater and separation time longer. Product and substrate were detected, quantified, and imaged simultaneously on the TLC plate using an Ambis (San Diego, CA) model 4000 Radioisotope Image Acquisition and Analysis System.

Assay of the GACC-Forming Activity

The assays were performed as described above for the ACC N-malonyltransferase and contained, in a volume of 50 μL: enzyme, 100 mM Tris-Cl, pH 8.0, 1 mM DTT, 1 mM EDTA, 2 mM GSH, and 2 mM [2,3-14C]ACC (270 nCi/assay). For some experiments, DTT and EDTA were omitted during preparation of the protein extracts and in the assays with no effect on specific activity of the enzyme. [14C]GACC and [14C]ACC were separated by TLC, detected, and quantified as described above for assay of the ACC N-malonyltransferase.

Purification and Hydrolysis of GACC

To isolate microgram quantities of MACC or GACC, the assays for ACC N-malonyltransferase and the GACC-forming activity were allowed to proceed for 6 to 8 h to completion. ACC N-malonyltransferase, purified as described by Martin and Saftner (1995), or GACC-forming enzyme, purified 1000-fold by ammonium sulfate precipitation followed by anion-exchange chromatography, was used to ensure that only GACC or MACC was formed in each assay. In such assays, as much as 99% of the ACC was converted to MACC, or as much as 75% of the ACC was converted to GACC. Since a high percentage of the ACC was converted to product, it was unlikely that GACC was a metabolite of a contaminant of the ACC. Assays were terminated and preparatively chromatographed using the conditions described above for the reversed-phase C18 system. The radiolabeled bands containing GACC or MACC were scraped from the TLC plates and eluted with methanol. For acid hydrolysis, aliquots of each sample were transferred to 10- × 70-mm ignition tubes and evaporated to dryness, and the residue was dissolved in 2 N HCl. The contents of the ignition tubes were frozen in liquid nitro-
gen, and the tubes were repeatedly flushed with nitrogen and then were sealed by heating while under vacuum. Control tubes containing an equal amount of [14C]ACC were treated in the same manner. The sealed tubes were heated at 100°C for 2 h, after which the HCl was evaporated. For base hydrolysis, aliquots of each sample in methanol were transferred to Teflon polytetrafluoroethylene vials, the solvent was removed, and 1 N NaOH was added. After 2 h at room temperature, the samples were neutralized with 1 N HCl. Acid- and base-hydrolyzed samples were analyzed using both the reversed-phase C18 TLC and the silica gel HPTLC-GHLF systems described above.

Derivatization of GACC and Analysis by GC-MS

GACC and MACC were purified as described above. The N-acetyl GACC dimethyl ester and the N-acetyl methyl ester of ACC and the dimethyl ester of MACC were prepared using essentially the procedure described by Michalczuk et al. (1992) for the derivatization of Trp. Putative MACC and GACC and synthetic ACC (Sigma) were dehydrated by multiple distillations in vacuo, first with absolute ethanol and then with dichloromethane. Immediately after distillation, 1 mL of anhydrous methanol (dehydrated by distillation from magnesium activated with iodine) and 0.5 mL of acetic anhydride were added, and the flask was closed tightly with a Teflon polytetrafluoroethylene-sleeved glass stopper and clip. The contents were mixed and placed in a heating block at 65°C. After 1 h, the methanol and acetic anhydride were evaporated in vacuo. GC-MS analysis was performed on a Hewlett-Packard 5890 gas chromatograph connected to a 5917A mass-selective detector. The gas chromatograph was equipped with a 15-m x 0.237-mm DB-1701 fused-silica capillary column (J & W Scientific, Folsom, CA), and helium was used as carrier gas at 1 mL/min. GC conditions were as follows: injector temperature, 250°C; initial oven temperature, 140°C for 2 min; followed by a ramp, 30°C/min up to 280°C.

Ethylene Treatment

For ethylene treatment, whole fruit were sealed for 12 to 16 h in a 37-L jar containing 120 µL/L ethylene in air and 50 mL of 10% KOH in a beaker to trap CO2. Controls were maintained in open air or enclosed in a jar with air only and 50 mL of 10% KOH and 50 mL of 0.25 M Hg(ClO4)2 to trap ethylene. All treatments were at 20°C in the dark.

RESULTS

Resolution of a New Conjugate of ACC

We previously described a new and facile assay for ACC N-malonyltransferase and showed the resolution by silica gel HPTLC of the substrate, [14C]ACC, and a single 14C-labeled product (presumably MACC) formed during incubation of [14C]ACC and malonyl-CoA with protein extracts from tomato seeds (Martin and Saftner, 1995). When crude protein extracts were used in the assays, a large amount of the product was formed in the absence of malonyl-CoA. Reactions using dialyzed, ammonium sulfate-precipitated protein were absolutely dependent on malonyl-CoA, but as much as 50% of the activity originally present in crude protein extracts was lost.

When the same ACC N-malonyltransferase assays were analyzed by reversed-phase C18 TLC (Fig. 1), one product was resolved in the absence of malonyl-CoA (lane 2) and two products were resolved in the presence of malonyl-CoA (lane 3), suggesting that the product formed in the absence of malonyl-CoA was not MACC. Lane 1 shows that no product formation occurred with heat-denatured enzyme. When dialyzed, ammonium sulfate-precipitated proteins were used, one malonyl-CoA-dependent product (MACC) was resolved (lane 5). The formation of the other product (GACC) did not occur with or without malonyl-CoA (lanes 4 and 5). GACC formation was recovered when crude supernatant (prepared as described in “Materials and Methods”) was added back to the assay (lane 6). The supernatant from acetone- or ethanol-precipitated protein was used for reconstitution, because of the ease of removal of the organic phase after precipitation of the proteins. Acetone- or ethanol-precipitated proteins, like ammonium sulfate-precipitated proteins, were unable to catalyze formation of GACC without reconstitution with crude supernatant. These results indicate that crude extracts contain a substrate and/or cofactor required for the enzyme-catalyzed formation of GACC from [14C]ACC.

Figure 1. Radioisotope image showing the separation of [14C]ACC, [14C]MACC, and a second 14C-labeled product (GACC) on a reversed-phase C18 TLC following assays of ACC N-malonyltransferase activity in extracts from tomato seeds. Assays were performed as described in “Materials and Methods” with the modifications noted for each lane number: lane 1, heat killed crude protein extract, ACC, and malonyl-Co A; lane 2, crude protein extract and ACC; lane 3, crude protein extract, ACC, and malonyl-Co A; lane 4, ammonium sulfate-precipitated protein and ACC; lane 5, ammonium sulfate-precipitated protein, ACC, and malonyl-Co A; lane 6, ammonium sulfate-precipitated protein, ACC, and the crude supernatant from acetone-precipitated protein; and lane 7, ammonium sulfate-precipitated protein, ACC, and 0.5 mM GSH.
Substrate and/or Cofactor Requirement for GACC-Forming Activity

Readily available potential cofactors/substrates were tested for the ability to substitute for crude supernatant in the assay. ATP, ADP, FAD, flavin mononucleotide, NADP⁺, NADPH, NAD⁺, NADH, and pyridoxal phosphate at 0.5 mM failed to support formation of GACC (Table I). Likewise, several CoA derivatives, including acetyl-CoA, succinyl-CoA, and n-butyryl-CoA, failed to support GACC formation (data not shown). However, 0.5 mM GSH supported GACC formation at a level higher than that observed by adding back crude supernatant (Table I). The product formed in the presence of crude supernatant and the product formed in the presence of GSH co-migrated on reversed-phase C₁₈ TLC (Fig. 1, lanes 6 and 7) and on normal-phase TLC (data not shown). When acetone-precipitated protein extracts were preincubated for 15 min with the cofactors listed in Table I prior to initiation of the reaction by the addition of GSH followed by [¹⁴C]ACC, no further stimulation or inhibition was observed beyond that observed with GSH alone (Table I), suggesting that these compounds probably do not serve as allosteric effectors or cofactors in the GACC-forming reaction. A number of divalent metals, including Mg²⁺, Mn²⁺, Zn²⁺, Ca²⁺, Fe²⁺, and Cu²⁺ supplied as either the sulfate or chloride salts at 0.5 or 5 mM, also did not support formation of GACC. Likewise, when added to assays containing GSH and ACC, these salts of divalent metals did not further stimulate formation of GACC. These results suggest that GSH and ACC are necessary and sufficient for the enzymatic formation of GACC.

Role of GSH and Identification of GACC

Several approaches were taken to elucidate the role of the GSH tripeptide in GACC formation, to determine the structure of GACC, and to identify the enzyme catalyzing its formation. They included determining the effect of reductants other than GSH, determining the fate of radiolabeled GSH and GACC analogs, establishing the type of bond between ACC and GSH, and GC-MS analysis of GACC. A number of other reductants at 5 mM, including ascorbate, Cys, 2-mercaptoethanol, DTE, DTI, and DTT in combination with EDTA, failed to substitute for GSH in stimulating GACC formation (data not shown). Although these results were obtained without DTT and EDTA in the extraction and dialysis buffers, their presence or absence did not affect the GACC-forming activity, suggesting that reduced sulfhydryl groups are not important for the activity of this enzyme.

The two commercially available radiolabeled forms of GSH, [³⁵S-Cys]GSH and [³⁵H-2-Gly]GSH, were used to further elucidate the role of GSH in GACC formation. Results are shown in Figure 2 for [³⁵S-Cys]GSH. Lane 3 shows the resolution of [¹⁴C]ACC and [¹⁴C]GACC by reversed-phase C₁₈ TLC in assays containing unlabeled GSH. Controls using heat-denatured enzyme and no GSH are shown in lanes 1 and 2, respectively. In lanes 4 to 6, [³⁵S-Cys]GSH was substituted for unlabeled GSH and unlabeled ACC was substituted for [¹⁴C]ACC in assays otherwise identical with those in lanes 1 to 3. Lane 6 shows that the ³⁵S-radiolabeled products did not co-migrate with GACC. Identical results were obtained when [³⁵H-2-Gly]GSH was substituted for [³⁵S-Cys]GSH (data not shown). These results show that neither Cys nor Gly is transferred to ACC during GACC formation. Since GSH radiolabeled in the Glu is not commercially available, we could not show by this method that Glu is, indeed, transferred to ACC upon GACC formation. Analogs of GSH and other peptides were used to further delineate what portion of the GSH tripeptide is required for GACC formation. GSSG and S-substituted GSls, all lacking the free reduced sulfhydryl group, were nearly as effective as GSH as substrates, providing further evidence that the sulfhydryl group is not involved in GACC formation (Table II). A number of dipeptides with γ-L-Glu at the N-terminal position, including those lacking Cys, served as substrates for GACC formation (Table III). Several were nearly as effective as GSH. Thus, Gly is not required, and a number of other amino acids can substitute for Cys in GACC formation. Several dipeptides and tripeptides lacking the N-terminal γ-L-Glu did not serve as substrates (Table IV). These included peptides with an N-terminal α-linked L-Glu.

Amide bonds are hydrolyzed by treatment with 2 N HCl for 3 h at 100°C, and, in fact, such treatment has been used routinely to cleave the amide linkage in MACC (Hoffman et al., 1983). Recovery of ACC can be demonstrated by co-chromatography with synthetic ACC and by chemical oxidation of ACC to ethylene as described by Lizada and Yang (1979). To test the possibility that GACC is an amide-linked conjugate between ACC and Glu, both GACC and

### Table I. Effect of potential substrates and cofactors on GACC formation

<table>
<thead>
<tr>
<th>Addition</th>
<th>GACC Formationa</th>
<th>Noneb</th>
<th>Plus GSHc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol min⁻¹ mg⁻¹ protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.91</td>
<td>84.8</td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>39.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>1.3</td>
<td>87.9</td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>1.3</td>
<td>91.8</td>
<td></td>
</tr>
<tr>
<td>NADPH</td>
<td>1.1</td>
<td>92.0</td>
<td></td>
</tr>
<tr>
<td>NADP⁺</td>
<td>1.9</td>
<td>82.8</td>
<td></td>
</tr>
<tr>
<td>NADH</td>
<td>1.6</td>
<td>84.5</td>
<td></td>
</tr>
<tr>
<td>NAD⁺</td>
<td>1.5</td>
<td>85.4</td>
<td></td>
</tr>
<tr>
<td>Pyridoxal phosphate</td>
<td>1.4</td>
<td>80.0</td>
<td></td>
</tr>
<tr>
<td>FAD</td>
<td>1.9</td>
<td>70.3</td>
<td></td>
</tr>
<tr>
<td>Flavin mononucleotide</td>
<td>4.7</td>
<td>83.0</td>
<td></td>
</tr>
<tr>
<td>GSH</td>
<td>85.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Acetone-precipitated protein from the seeds of ripe tomato fruit was prepared as described in "Materials and Methods." b Assays containing protein, 100 mM Tris-Cl, pH 8.0, 2 mM EDTA, 1 mM DTT, and 0.5 mM cofactor were initiated with 2 mM [¹⁴C]ACC. c Assays containing protein, 100 mM Tris-Cl, pH 8.0, 2 mM EDTA, 1 mM DTT, 2 mM GSH, 2 mM [¹⁴C]ACC, and 0.5 mM cofactor. Protein was incubated for 15 min with the cofactor, prior to addition of GSH, followed by [¹⁴C]ACC to initiate the reaction. d Not determined.
Table II. S-Substituted GSH analogs serve as donors for GACC formation

<table>
<thead>
<tr>
<th>Donor</th>
<th>Percentage of Activity with GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>100</td>
</tr>
<tr>
<td>γ-Glu-Cys</td>
<td>80</td>
</tr>
<tr>
<td>γ-Glu-Gly</td>
<td>3</td>
</tr>
<tr>
<td>γ-Glu-Gln</td>
<td>72</td>
</tr>
<tr>
<td>γ-Glu-Leu</td>
<td>91</td>
</tr>
<tr>
<td>γ-Glu-Phe</td>
<td>65</td>
</tr>
<tr>
<td>γ-Glu-Trp</td>
<td>95</td>
</tr>
<tr>
<td>γ-Glu-Glu</td>
<td>50</td>
</tr>
<tr>
<td>γ-Glu-His</td>
<td>75</td>
</tr>
</tbody>
</table>

MACC were purified as described in “Materials and Methods” and were subjected to acid hydrolysis. Chromatography of samples before and after hydrolysis on both silica gel (Fig. 3B) and reversed-phase C18 (Fig. 3A) TLC showed that acid-hydrolyzing conditions did not affect the migration of ACC and that the radioactivity released from both GACC and MACC by hydrolysis co-migrated with ACC. In contrast, treatment of the two conjugates for 2 h with 1 N NaOH (conditions that should hydrolyze ester-linked conjugates) resulted in recovery of compounds that co-migrated with GACC or MACC on both TLC systems (data not shown).

These results taken together suggest that GACC is 1-(γ-L-glutamylamino)cyclopropane-1-carboxylic acid and that the formation of GACC is catalyzed by a γ-glutamyltranspeptidase. The electron impact mass spectrum of derivatized GACC, shown in Figure 4, further supports this conclusion. A molecular ion at 300, a molecular ion minus a proton at 299, and a fragment at 187 were consistent with an N-acetyl GACC dimethyl ester and the Glu fragment, respectively. The structure of GACC and the reaction catalyzing its formation is shown in Figure 5.

Enzyme Kinetics

Separation by normal-phase silica gel TLC followed by radioisotope imaging now forms the basis for a facile method of assay of ACC N-malonyltransferase activity (Martin and Saftner, 1995). The same methodology has been applied effectively to the measurement of the GACC-forming activity. The substitution of the reversed-phase C18 TLC for normal-phase silica gel TLC permitted measurement of both activities simultaneously. In this assay, GACC-forming activity, like the ACC N-malonyltransferase activity, is linear with time for nearly 2 h (data not shown). The rate of formation of GACC showed saturation kinetics with increasing concentration of GSH (Fig. 6A). Lineweaver-Burk plots showed a Km for GSH of 175 μM (Fig. 6A, inset). The rate of formation of GACC showed biphasic kinetics with respect to ACC concentration, and the Km values were estimated to be 150 μM and 1.0 mM.

Table III. Several dipeptides with γ-L-glutamyl N-terminal substituents serve as donors for GACC formation

<table>
<thead>
<tr>
<th>Donor Percentage of Activity with GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH (γ-Glu-Cys-Gly)</td>
</tr>
<tr>
<td>γ-Glu-Cys</td>
</tr>
<tr>
<td>γ-Glu-Gly</td>
</tr>
<tr>
<td>γ-Glu-Gln</td>
</tr>
<tr>
<td>γ-Glu-Leu</td>
</tr>
<tr>
<td>γ-Glu-Phe</td>
</tr>
<tr>
<td>γ-Glu-Trp</td>
</tr>
<tr>
<td>γ-Glu-Glu</td>
</tr>
<tr>
<td>γ-Glu-His</td>
</tr>
</tbody>
</table>

Table IV. Peptides lacking an N-terminal γ-linked Glu do not serve as donors for GACC formation

<table>
<thead>
<tr>
<th>Donor Percentage of Activity with GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
</tr>
<tr>
<td>Cys-Gly</td>
</tr>
<tr>
<td>Glu-Glu</td>
</tr>
<tr>
<td>Glu-Ala</td>
</tr>
<tr>
<td>Glu-Val-Phe</td>
</tr>
<tr>
<td>Glu-Gly-Phe</td>
</tr>
<tr>
<td>Pro-Gly</td>
</tr>
<tr>
<td>Gly-Ala</td>
</tr>
</tbody>
</table>

Assays were performed as described in “Materials and Methods,” except di- or tripeptides at 2 mM replaced GSH. Enzyme was extracted from the seeds of ripe fruit and was purified approximately 1000-fold to a specific activity of 80,000 pmol min⁻¹ mg⁻¹ protein.

Figure 2. Radioisotope image showing the resolution of ACC, GSH, and GACC by reversed-phase C18 TLC. Lane 1, Heat-denatured enzyme, [14C]ACC, and unlabeled GSH; lane 2, enzyme and [14C]ACC; lane 3, enzyme, [14C]ACC, and unlabeled GSH; lane 4, heat-denatured enzyme, unlabeled ACC, and [35S-Cys]GSH; lane 5, enzyme and [35S-Cys]GSH; lane 6, enzyme, unlabeled ACC, and [35S-Cys]GSH. Assays were performed as described in “Materials and Methods” except where indicated that unlabeled ACC was substituted for [14C]ACC and [35S-Cys]GSH. Assays were performed as described in “Materials and Methods” except dipeptides at 2 mM replaced GSH. Enzyme was extracted from the seeds of ripe fruit and was purified approximately 1000-fold to a specific activity of 80,000 pmol min⁻¹ mg⁻¹ protein.
Figure 3. Radioisotope image showing the resolution by reversed-phase C18 TLC (A) and silica gel HPTLC (B) of ACC (lane 1), ACC subjected to acid-hydrolyzing conditions (lane 2), GACC (lane 3), GACC after acid hydrolysis (lane 4), MACC (lane 5), and MACC after acid hydrolysis (lane 6). Conditions for GACC and MACC synthesis, purification, acid hydrolysis, and chromatographic separation are described in "Materials and Methods."

Developmental and Spatial Pattern of the GACC-Forming Activity

The GACC-forming activity was measured in extracts of seeds, pericarp, placenta, and locular fluid from tomato fruit at several stages of development (Fig. 7). The GACC-forming activity was approximately 1500 pmol min⁻¹ g⁻¹ fresh weight in the pericarp of immature green fruit at 7 to 10 d postanthesis and increased gradually through fruit development to a plateau in orange to ripe fruit of approximately 5000 pmol min⁻¹ g⁻¹ fresh weight (Fig. 7A). A similar level of GACC-forming activity and a similar pattern of expression were observed in the placenta. In the locular fluid, the GACC-forming activity was approximately 10-fold lower in immature green fruit, but as was shown in the pericarp and placenta, activity increased 3- to 4-fold throughout the course of fruit development and ripening (Fig. 7A). Changes in GACC-forming activity in the seed were small (approximately 1.5-fold) throughout fruit development and seed maturation (Fig. 7B). However, at all stages of development, the GACC-forming activity was 3- to 10-fold higher in the seed than in the maternal tissues.

Effect of Ethylene on the GACC-Forming Activity

Treatment of whole fruit with 120 μL/L ethylene for 12 to 16 h stimulates ACC N-malonyltransferase activity in extracts of pericarp from preclimacteric fruit by as much as 75- to 100-fold (Liu et al., 1985; Martin and Saftner, 1995). In contrast, ethylene treatment of the fruit failed to alter the in vitro γ-glutamyltranspeptidase activity in the pericarp of preclimacteric fruit (Fig. 8). In climacteric fruit, treatment with ethylene resulted in a 2- to 3-fold decrease in activity.

DISCUSSION

Identification of a New ACC-Conjugating Activity

The neutral amino acid ACC has previously been shown to undergo only two reactions in plants: malonyl-CoA-dependent malonylation catalyzed by ACC N-malonyltransferase (Amrhein et al., 1981) and Fe²⁺- and ascorbate-dependent oxidation to ethylene catalyzed by ACC oxidase (Vereridis and John, 1991). In a Pseudomonas species and in the yeast Hansenula saturnus ACC also is oxidatively deaminated to α-ketobutyrate and ammonia in a pyridoxal phosphate-dependent reaction (Walsh et al., 1981; Honma, 1985). We describe the identification in tissue extracts of tomato fruit of a γ-glutamyltranspeptidase activity, which catalyzes, in a glutathione-dependent reaction, the formation of GACC, a new conjugate of ACC.

The tripeptide γ-glutamylcysteinylglycine (GSH) has been shown to participate in three major classes of reactions. In these reactions GSH serves as (a) a hydrogen donor for reductase reactions, (b) a donor of GSH in reactions catalyzed by GST, and (c) a γ-glutamyl donor or a γ-glutamylcysteine donor in transpeptidase reactions catalyzed by γ-glutamyl transpeptidase or γ-glutamylcysteine dipeptidyl transpeptidase. Using a multifaceted approach, we systematically examined the role for GSH in GACC biosynthesis.

Presumably, to serve as a reductant, the sulfhydryl group on GSH must be free and reduced. However, GSSG, S-substituted analogs of GSH, and peptides lacking a sulfhydryl group are effective substrates for GACC formation (Tables II and III). Thus, GSH is not serving as a source of reductant in GACC formation.

GST-catalyzed conjugation reactions are ubiquitous in bacteria, fungi, animals, and plants (Pickett and Lu, 1989), and plant GSTs are receiving increased attention in relation to their induction by herbicides, herbicide safeners, and pathogen attack and their role in conjugation and detoxification of herbicides (Meyer and Goldsbrough, 1991; Fuerst et al., 1993; Irzyk and Fuerst, 1993; Mauch and Dudler, 1993). GSTs catalyze the nucleophilic addition of GSH through a thioether linkage to hydrophobic acceptors containing electrophilic centers, such as aryl and alkyl halides, olefins, organic hydroperoxides, quinones, alkenes, and
A New ACC-Conjugating Activity in Tomato Fruit

Figure 4. The electron impact mass spectrum of the N-acetyl GACC dimethyl ester shows a molecular ion at 300, the molecular ion minus a proton at 299, and the Glu fragment at 187. GACC was purified by reversed-phase C18 chromatography and derivatized as described in "Materials and Methods." GC-MS was performed on a Hewlett-Packard 5890 gas chromatograph connected to a 5971A mass-selective detector as described in "Materials and Methods." The gas chromatograph was equipped with a 15-m × 0.22-mm DB-1701 fused silica capillary column (J & W Scientific). Helium at 1 mL/min was used as the carrier gas.

Figure 5. A γ-glutamyltranspeptide catalyzes the transfer of the Glu in the GSH tripeptide to ACC with the formation of GACC.

epoxides (Douglas, 1988; Pickett and Lu, 1989). Although the substrate specificity of GSTs is very broad, ACC is not a likely GST substrate. GSTs catalyze the transfer of the entire GSH tripeptide and require that the sulfhydryl group be free. GACC formation occurred when GSSG, S-substituted analogs of GSH lacking a free sulfhydryl group, or other peptides lacking a sulfhydryl group were substituted for GSH (Tables II and III). Furthermore, when using radiolabeled GSH, the Cys and Gly in the GHS tripeptide were not transferred to ACC (Fig. 2). Thus, our results also show that GSH is not forming a conjugate with ACC in a GST-catalyzed reaction.

ACC is a much more likely substrate for a transpeptidase reaction, such as that catalyzed by γ-glutamyl transpeptidase or γ-glutamylcysteine dipeptidyl transpeptidase. These two enzymes catalyze, respectively, the cleavage of cysteinylglycine or Gly from the GSH tripeptide and the transfer of the γ-glutamyl or γ-glutamylcysteine moieties to an amino acid or dipeptide, forming an amide linkage between the acceptor and the γ-carboxyl group of Glu or the α-amino group of Cys. Since our results show that Cys and Gly are not transferred from GSH to form GACC (Fig. 2), a γ-glutamylcysteine dipeptidyl transpeptidase reaction can be ruled out. The ability of the GACC-forming activity to use GSSG, S-substituted GSHs, and a number of di- and tripeptides having N-terminal γ-linked Glu and the inability to use peptides lacking N-terminal γ-linked Glu are consistent with the substrate requirements for γ-glutamyltranspeptidases from mammalian sources (Tate and Meister, 1974; Meister and Tate, 1976). Thus, our results support
the conclusion that GACC is 1-(y-L-glutamylamino)cyclopropane-1-carboxylic acid and that its formation is catalyzed by a y-glutamyltranspeptidase. GC-MS analysis of the N-acetyl GACC dimethyl ester confirmed this conclusion (Fig. 4).

\( \gamma \)-Glutamyltranspeptidase has been studied extensively in bacteria in which the enzyme is reported to be localized in the periplasmic space (Suzuki et al., 1986) and in animals in which the enzyme is most often localized on the external surface of the plasma membrane (Meister et al., 1981). In animals, this class of enzyme is involved in amino acid transport, GSH degradation and Cys recycling, and processing of S-conjugates of GSH (Tsao and Curthoys, 1980; Inoue et al., 1983). \( \gamma \)-Glutamyl amino acids and \( \gamma \)-glutamyltranspeptidase activity have been reported to occur in plants, but the function and in vivo substrate(s) are unknown (Kasai, 1982; Lancaster and Shaw, 1994).

**Regulation of ACC Conjugation**

Many plant tissues contain pools of ACC and conjugated ACC and synthesize at least a low level of ethylene. Factors regulating this balance are incompletely understood. In the tomato fruit, the internal ethylene concentration, the ethylene evolution rate, the ACC synthase activity, and the pool of ACC were all shown to be low in the pericarp of mature green fruit but to increase dramatically at the breaker stage of ripening (Hoffman and Yang, 1980; Kende and Boller, 1981; Su et al., 1984). Why conjugated ACC steadily increased throughout mature green stages when ACC and ethylene levels were low, followed by a rapid burst of conjugate formation at the same time as climacteric ethylene biosynthesis, is unclear (Su et al., 1984). Su et al. (1984) suggested that conjugation served to modulate ethylene biosynthesis but that the need for modulation at these stages of fruit development was not clear.

In this paper and in our prior work (Martin and Saffert, 1995), we described the developmental and tissue-specific patterns of two enzymatic activities in extracts from tomato maternal tissues and seeds that are capable of catalyzing the conjugation of ACC. Our results suggest that, at least in the pericarp, ACC N-malonyltransferase activity, which catalyzes the formation of MACC, is regulated by ethylene. ACC N-malonyltransferase activity was barely
Whole fruit at each developmental stage were harvested and exposed to air or ethylene (120 μL/L) in air for 12 to 16 h. Pericarp was harvested and extracted, and enzyme activity was measured as described in “Materials and Methods.” The activity at each stage is the average of the activity obtained for four to five fruit harvested at the same time. Assays were performed in triplicate. Activities during different growing seasons were measured but not averaged. G F WT, Grams fresh weight; IMM, immature; MAT, mature.

detectable in preclimacteric fruit, increased severalfold at the breaker stage, and subsequently declined in a manner that paralleled the increase and subsequent decrease in climacteric ethylene biosynthesis. Likewise, ethylene treatment of preclimacteric, but not climacteric, fruit resulted in a dramatic increase (as much as 50- to 100-fold) in the ACC N-malonyltransferase activity (Liu et al., 1985; Martin and Saftner, 1995). In contrast, the γ-glutamyltranspeptidase activity increased severalfold at the breaker stage, and subsequently declined in a manner average of the activity obtained for four to five fruit harvested at the same time. Assays were performed in triplicate. Activities during different growing seasons were measured but not averaged. G F WT, Grams fresh weight; IMM, immature; MAT, mature.

Figure 8. Effect of ethylene treatment on the GACC-forming activity. Whole fruit at each developmental stage were harvested and exposed to air or ethylene (120 μL/L) in air for 12 to 16 h. Pericarp was harvested and extracted, and enzyme activity was measured as described in “Materials and Methods.” The activity at each stage is the average of the activity obtained for four to five fruit harvested at the same time. Assays were performed in triplicate. Activities during different growing seasons were measured but not averaged. G F WT, Grams fresh weight; IMM, immature; MAT, mature.

Detection of the endogenous levels of ACC and both of the conjugates of ACC will be the next step in elucidating their role in fruit and seed development. Methodology previously used for measuring endogenous pools of ACC and conjugated ACC presumed the presence of a single acid-hydrolyzable, amide-linked conjugate. We are now developing the GC-MS methodology necessary to identify and quantify endogenous pools of both conjugates simultaneously. We have already shown that conjugates that co-migrate with the GACC and MACC are also formed in vivo by tomato fruit discs incubated with [14C]ACC (M.N. Martin, J.D. Cohen, and J.P. Slovin, unpublished results), but such a system cannot be extrapolated to predict the level of these conjugates in the whole fruit.

ACKNOWLEDGMENTS

The authors wish to thank Dr. George Buta for generously sharing his laboratory facilities and scientific expertise and Dr. Janet Slovin for critically reading the manuscript.

Received May 18, 1995; accepted August 12, 1995.
Copyright Clearance Center: 0032-0889/95/109/0917/10.

LITERATURE CITED


