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Subcellular localization of 1-aminocyclopropane-1-carboxylate oxidase in tomato cells

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Abstract. The localization of 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase was examined in suspension-cultured cells of tomato (*Lycopersicon esculentum* Mill.), using cell-fractionation techniques, followed by immunoblot analysis with monospecific antibodies raised against a tomato ACC oxidase expressed in *Escherichia coli*. When assayed in vivo, ACC oxidase had a low activity in untreated tomato cells but was strongly induced when the cells were supplied with its substrate, ACC. Immunoblots showed that this induction was accompanied by the accumulation of a single protein corresponding to ACC oxidase, with an apparent molecular mass (M_r) of 36 kDa. The level of this protein in induced cells, estimated by immunoblotting, was compared with that in protoplasts and vacuoles, and with that in various particulate and soluble fractions obtained by differential centrifugation of cell homogenates. It was found that the ACC oxidase antigen was absent from the vacuole, and that most of it was localized in the cytoplasm of the protoplasts without being associated with membranes. Measurements of ACC oxidase activity in preparations of protoplasts and vacuoles supported these results.

Key words: 1-Aminocyclopropane-1-carboxylate oxidase – Ethylene biosynthesis – *Lycopersicon*– Subcellular localization – Vacuole

The plant hormone ethylene is synthesized from 1-aminocyclopropane-1-carboxylic acid (ACC). As reviewed recently (Kende 1993), the identity of the "ethylene-forming enzyme" remained elusive for a long time after discovery of ACC. Most of the cell-free systems initially reported to convert ACC to ethylene lacked the stereospecificity with regard to 1-butene production from 2-ethyl-derivatives of ACC observed in vivo (Yang and Hoffman 1984). However, preparations of vacuoles from pea and *Vicia* retained the capacity to release ethylene from ACC with the correct stereospecificity (Guy and Kende 1984). This activity required membrane integrity (Mayne and Kende 1986), suggesting a tonoplast localization, but appeared to be independent of the membrane potential (Guy 1990). Experiments of Porter et al. (1986) and of Erdman et al. (1989) also indicated a vacuolar localization of the enzyme converting ACC to ethylene. In contrast, Bouzayen et al. (1990), studying conversion of radiolabelled ACC, concluded that tissue-cultured *Vitis* cells had both an internal and external site of ethylene biosynthesis.

Attempts to detect authentic ACC oxidase activity in soluble extracts initially failed since the enzyme activity appeared to be extremely unstable in extracts of many plants, thus preventing in vitro studies and the purification of the enzyme. However, more recently, authentic ACC oxidase could be measured in cell-free, soluble preparations of certain fruit tissues (review: Kende 1993). These studies suggest that at least some ACC oxidases are soluble enzymes but they do not address the question of subcellular localization.

The cloning and functional expression of ACC oxidase from tomato (Hamilton et al. 1991; Spanu et al. 1991) opened a new way to look at localization of ACC oxidase, using antibodies against ACC oxidase produced in bacteria. When ACC oxidase from tomato was expressed in yeast, the enzyme was not located in the vacuoles although it appeared to be associated with a particulate fraction (Peck et al. 1992). More recently, immunocytochemical experiments indicated that the ethylene-forming enzyme in ripening tomato was to a large extent extracellular (Rombaldi et al. 1994).

Here, we examine the localization of ACC oxidase in suspension-cultured tomato cells, and we demonstrate that in this tissue, in contrast to ripening fruit (Rombaldi et al. 1994), the enzyme is located intracellularly, and that it is absent from vacuoles, as in yeast (Peck et al. 1992),

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Abbreviations: ACC = 1-aminocyclopropane-1-carboxylic acid

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but does not show any association with a particulate fraction, indicating that it is a soluble cytoplasmic enzyme.

Plant material and treatments. A suspension culture of tomato cells (*Lycopersicon esculentum* Mill., line Msk8, kindly provided by M. Koornneef, The Agricultural University, Wageningen, The Netherlands) was grown on a rotary shaker (120 cycles per minute) and used 7–9 d after subcultivation (details: Felix et al. 1991). To induce ACC oxidase, aliquots of 1.3 ml of the cell suspension were incubated with 1 mM ACC in 6-ml glass tubes for the times indicated, putting the tubes, either open or closed with a rubber septum, in a rack fixed in a tilted position on the rotary shaker. For preparation of protoplasts and vacuoles and for differential-centrifugation experiments, aliquots of 10 ml of the cell suspension were pretreated overnight with 1 mM ACC in closed 50-ml Falcon tubes under sterile conditions.

Polyclonal antibodies specific for ACC oxidase from tomato cells. Tomato ACC oxidase was expressed in *Escherichia coli* on the basis of a construct derived from pHTOM5, a cDNA isolated from the tomato cells used in the present work, as described before (Peck et al. 1992). Briefly, a fragment containing the full open reading frame except for the first 26 codons was cloned into a pDS/RBSII-2 expression vector and introduced into *E. coli* M15 (Hochuli et al. 1988) carrying the pDMI,1 plasmid for constitutive expression of the lac repressor. The ACC oxidase was induced in overnight cultures of the transformed *E. coli* by addition of 2 mM isopropyl-β-thiogalactoside and allowed to accumulate for 8 h. Inclusion bodies containing ACC oxidase were extracted from lysed bacteria and washed with detergent buffers (Harlow and Lane 1988). The washed inclusion bodies, which contained an almost pure protein with a molecular weight of approx. 34 kDa, were used for immunization of rabbits following established procedures (Harlow and Lane 1988; for details see Peck et al. 1992). The serum was used for immunoblots after four rounds of immunization.

Preparation and purification of protoplasts and sampling for immunoblots. Aliquots (10 ml) of cells treated overnight with ACC were collected by centrifugation at 200 · *g* for 10 min. The supernatant was removed and replaced by half the original volume (5 ml) of 0.8 M mannitol containing 4% (w/v) cellulase Y-23 and 0.3% (w/v) pectolyase Y-C (both from Seishin Pharmaceutical Company, Tokyo, Japan), 10 mM $CaCl_2$, and 50 mM Mes (2-(*N*-morpholino)ethanesulphonic acid) adjusted with Tris to pH 6.5, and brought to the original volume (10 ml) with water. At appropriate times, 250 µl samples were removed for immunoblot analysis, carefully mixed with 1 vol. of gradient buffer (0.4 M mannitol containing 5 mM $CaCl_2$ and 25 mM Mes, adjusted with Tris to pH 6.5) and 1 vol. of water, pelleted at 1500 · *g* for 5 min, and frozen in liquid nitrogen after removal of the supernatant. After 6 h, the remaining protoplast suspension (8 ml) was mixed with 2 ml Percoll in a 50-ml plastic tube. The suspension was overlayered with 5 ml of gradient buffer containing each 10% (w/v), 5% (w/v) and 0% (w/v) Percoll. The gradient was centrifuged for 15 min at 1500 · *g*. Protoplasts accumulating at the interfaces between 10% and 5% or 5% and 0% Percoll were collected. After carefully mixing with 1 volume of gradient buffer and 1 vol. of water, the protoplasts were pelleted for 15 min at 1500 · *g* and resuspended carefully in 1 ml of vacuole buffer (VB), consisting of 0.2 M mannitol with 20 mM Hepes, adjusted with Tris to pH 8.0, and 0.5 mM 3-([3-cholamidopropyl]-dimethylammonio)-2-hydroxy-1-propanesulphonate, and immediately frozen in liquid nitrogen or lysed for vacuole production. In the case of comparison of ACC oxidase activity in protoplasts and vacuoles, protoplasts were resuspended in VB with 0.3 M instead of 0.2 M mannitol concentration.

Preparation and purification of vacuoles. Vacuoles were isolated and purified essentially as described by Vögeli-Lange and Wagner (1990). One volume of VB containing 2 mM EDTA was added to purified protoplasts. The suspension was gently stirred with a toothpick for 5 min to release most of the vacuoles. The slimy aggregate of lysed cells was removed with the toothpick. The vacuole suspension was carefully mixed in a 15-ml Falcon tube with 1 vol. of VB with 0.4 instead of 0.2 M mannitol, containing 20% (v/v) Percoll, and overlayered with 1 ml each of VB with 0.3 M instead of 0.2 M mannitol, containing 5%, 2.5% and 0% (v/v) Percoll. This gradient was centrifuged for 10 min at 1500 · *g*. Vacuoles accumulated between the 2.5% and 0% or in the 0%-layer were collected and directly used for determination of ACC oxidase activity or frozen in liquid nitrogen for immunoblot analysis.

Sampling for immunoblots and determination of ACC oxidase activity. Samples (0.3 ml) of appropriately treated cell suspensions (2 ml) were pelleted (5 min at 1600 · *g*), and the cells were frozen in liquid nitrogen after removal of the supernatant. The cells remaining in the tubes were aerated with fresh air three times, supplemented with 1 mM ACC, and then shaken without caps for 10 min to remove any accumulated ethylene, and to allow uptake of ACC up to levels saturating ACC oxidase. Then, the tubes were closed with a rubber cap and incubated for 30 min, air (1 ml) was removed, and the ethylene level was determined by gas chromatography (Felix et al. 1991). For measuring ACC oxidase activity during protoplast preparation, samples of 1 ml of cell suspension pretreated with ACC were incubated with 4 ml air in syringes in the presence of 1 mM ACC, the cell-wall-degrading mixture, and 0.1 mM $FeSO_4$ and 10 mM Na-ascorbate where indicated. Air samples (1 ml) were removed, and the syringes were aerated, re-sealed and further incubated for the next measurement. In the case of protoplasts and vacuoles, ACC oxidase activity was assayed in 0.5 ml of VB with 0.3 M mannitol, containing 1 mM ACC. After 30 min of incubation without shaking in a 2-ml vial, 1 ml air was removed and assayed for ethylene.

Gel electrophoresis and immunoblotting. Cell pellets were thawed and resuspended in 50 µl of 2 × sample buffer (1 × sample buffer (SB) is 66 mM Tris(HCl), pH 6.8, 1.25% (w/v) dithiothreitol, 1.6% (w/v) SDS, 8% (v/v) glycerol and 0.01% (w/v) bromophenol blue), and boiled for 2 min. The SDS-PAGE on a 10% gel was carried out according to Laemmli (1970) in a minigel assembly (Bio-Rad, Glattbrugg, Switzerland). Immunoblot analysis was carried out according to Harlow and Lane (1988) using antiserum in a 1000-fold dilution.The lanes on immunoblots represent same amounts of cells.

Marker enzymes. α-Mannosidase activity was used as vacuolar marker (Boller and Kende 1979). Malate dehydrogenase (Bergmeyer 1974) was used as an extravacuolar marker.

Differential centrifugations. The procedure was modified from Peck et al. (1992). After overnight pretreatment with ACC, the cells were pelleted and resuspended with an equal volume of ice-cold extraction buffer (EB: 50 mM Mes, adjusted with Tris to pH 7.8, 2 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride and 250 mM sucrose) and blended thoroughly in a glass homogenizer (Potter-Elvehjem, Zürich, Switzerland) on ice. The homogenate, labelled H, was centrifuged at 100 · *g* for 15 min. The pellet was washed three times with 10 vol. of ice cold EB and once in water to yield pellet P1, while the resulting supernatant S1 was further centrifuged at 18500 · *g* for 30 min to yield pellet P2. Centrifugation of the resulting supernatant S2 at 140000 · *g* for 1 h yielded pellet P3 and the soluble fraction S3. All centrifugations were carried out at 4°C. For immunoblot analysis, aliquots of H and S1-3 were mixed with 0.25 vol. of 5 × sample buffer and P1-3 were suspended in 1 × sample buffer.

Induction of ACC oxidase in tomato cells by ACC. 1-Aminocyclopropane-1-carboxylic acid oxidase in suspension-cultured tomato cells was studied by measurements of its activity in vivo and by immunoblot analysis with an antiserum raised against a tomato ACC oxidase produced in *E. coli*. Attempts to extract and measure

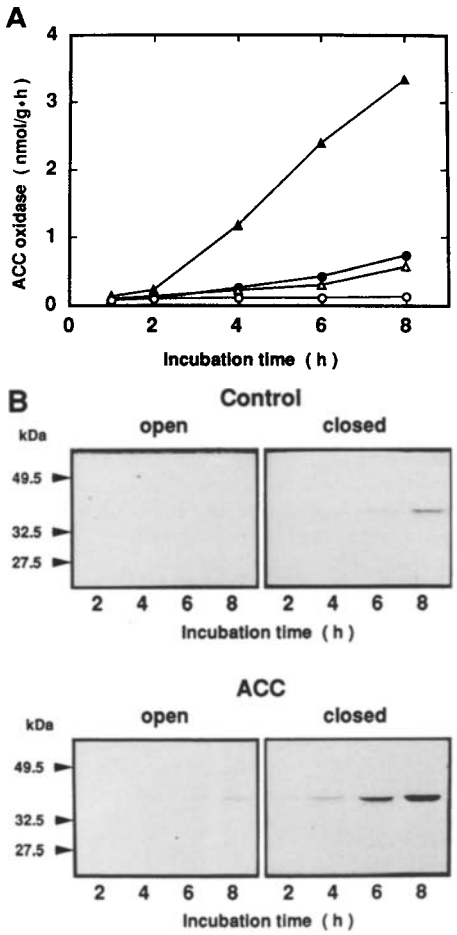

Fig. 1A, B. Induction of ACC oxidase in tomato cells during treatment with 1 mM ACC. **A** Time course of ACC oxidase activity during treatment of cells with ACC (*triangles*) or water (*circles*) in open tubes (*open symbols*) or closed tubes (*filled symbols*). Each point represents an independent sample. **B** Immunoblot analysis of extracts from the cells shown in **A**. Each lane represents the same amount of cells

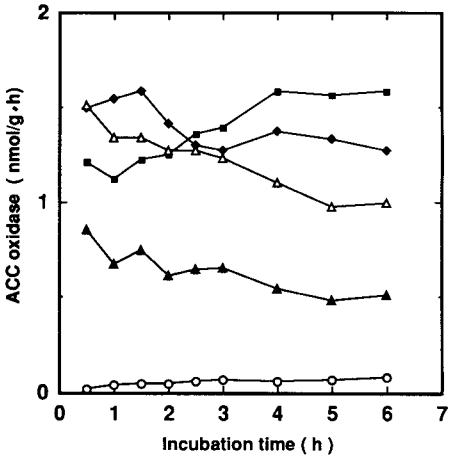

Fig. 2. 1-Aminocyclopropane-1-carboxylic acid oxidase activity in tomato cells during incubation with 0.4 M mannitol with and without cell-wall-digesting enzymes. Cells were pretreated overnight with water in open tubes (○) or with 1 mM ACC in closed tubes (*other symbols*). The cells were then further incubated in closed syringes with ACC (○, △), ACC and mannitol (▲), ACC and the complete cell-wall-digestion mixture (■) or ACC and digestion mixture and ascorbate/Fe (◆), ethylene production was measured in intervals for 6 h

ACC oxidase in vitro, following the procedures of Ververidis and John (1991), were unsuccessful. Untreated cells had a very low constitutive activity of ACC oxidase (Fig. 1A), as described previously (Felix et al. 1991; Spanu et al. 1991), and no detectable antigen corresponding to ACC oxidase (Fig. 1B). In initial experiments, ACC oxidase was induced by treatments with different elicitors, e.g. xylanase or fungal cell wall preparations (Felix et al. 1991), but the cell walls of elicitor-treated cells could not be digested in the usual protoplasting mixtures, thus preventing the production of protoplasts. This may be connected to the oxidative cross-linking of cell wall proteins (Bradley et al. 1992).

However, it was subsequently found that a treatment with ACC, the substrate of ACC oxidase, caused a strong increase of this enzyme, as measured in vivo (Fig. 1A) and led to the accumulation of a single immunoreactive polypeptide with an M_r of approx. 36 kDa, the molecular mass expected for ACC oxidase (Fig. 1B). This induction was weak if treatments were carried out in open tubes where ethylene diffused away but strong if they were done in closed tubes in which the ethylene produced by the cells was accumulating to high levels, e.g. to 33 $\mu l \cdot l^{-1}$ after 8 h of incubation in the experiment shown in Fig. 1A. At this stage, ACC oxidase was induced at least 20-fold above its level in control cells (Fig. 1A). It continued to increase and reached a plateau at levels of 100-fold above that in control cells after about 15 h (data not shown). In closed tubes, even control cells without added ACC accumulated 0.8 $\mu l \cdot l^{-1}$ ethylene during 8 h and showed some induction of ACC oxidase (Fig. 1A, B). These results indicate that ACC causes induction of ACC oxidase by way of ethylene.

Activity of ACC oxidase during preparation of protoplasts. Tomato cells treated in closed tubes with 1 mM ACC overnight exhibited very high levels of ACC oxidase and were thus used for localization studies. Protoplasts were prepared in a digestion mixture osmotically stabilized with 0.4 M mannitol, and the level of ACC oxidase was measured in vivo during the incubation (Fig. 2). In control cells incubated without any additions except for ACC, ACC oxidase decreased somewhat during the 6 h of incubation. In control cells treated with 0.4 M mannitol and ACC only, the activity of ACC oxidase fell immediately to about half the activity of untreated control cells and then declined in parallel with the untreated controls. The inhibitory effect of mannitol was almost completely reversed when the complete digestion mixture was added. Addition of $FeSO_4$ and sodium ascorbate did not change ACC oxidase activity significantly (Fig. 2).

After 6 h of incubation in the complete digestion mixture, cells were converted into round, osmotically sensitive protoplasts, indicating complete degradation of the cell walls. Measurement of ACC oxidase in vivo showed that the cells retained almost the full capacity of oxidizing

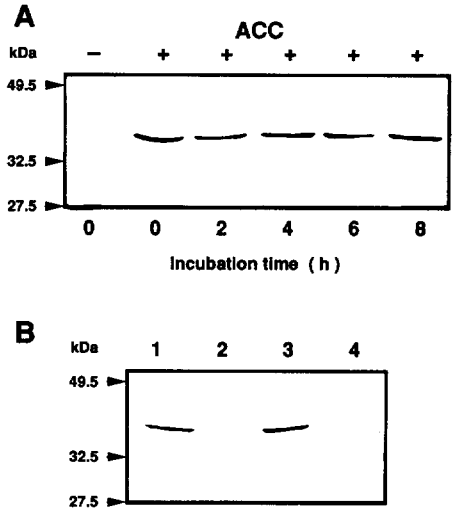

Fig. 3A, B. Immunoblot analysis of ACC oxidase in induced cells during preparation of tomato protoplasts and vacuoles. **A** preparation of protoplasts. The lanes represent extracts from cells pretreated overnight with water in open tubes (*first lane*) or 1 mM ACC in closed tubes (*second lane*) and from ACC-pretreated cells after 2, 4, 6 and 8 h of protoplast preparation. **B** Fractions of protoplasts and vacuoles. *Lane 1*, crude protoplasts; *lane 2*, digestion mixture after preparation of protoplasts; *lane 3*, purified protoplasts; *lane 4*, purified vacuoles. *Lanes 1, 3*, and *4* represent preparations with the same α-mannosidase activity. *Lane 2* represents the amount of digestion medium in which the protoplasts shown in *lane 1* were suspended

Table 1. Comparison of ACC oxidase activity in protoplasts and vacuoles of *L. esculentum* in relation to a vacuolar marker enzyme (α-mannosidase) and to an extravacuolar marker enzyme (malate dehydrogenase). Values for ACC oxidase and malate dehydrogenase refer to samples with the same α-mannosidase activity

Fraction	Relative activity of		
	α-Mannosidase	ACC oxidase	Malate dehydrogenase
Protoplasts	1	31.5	66.4
Vacuoles	1	5.18	10.2

ACC to ethylene during their conversion into protoplasts, i.e. within 6 h of incubation, indicating that the enzyme activity in vivo is quite stable during preparation of protoplasts. This is in contrast to the observations in other systems where ethylene-forming activity was strongly reduced during preparation of protoplasts (Mattoo and Lieberman 1977; Porter et al. 1986).

Subcellular localization of ACC oxidase. Immunoblot analysis demonstrated that the level of ACC oxidase antigen remained approximately constant during the procedure of protoplast preparation, and that the antigen was absent from the digestion mixture after preparation of protoplasts (Fig. 3). Thus, in the suspension-cultured tomato cells used in the present study, all the ACC oxidase appears to be inside the protoplast, and there is no indication of a cell wall localization of ACC oxidase.

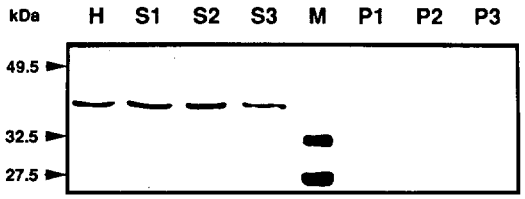

Fig. 4. Immunoblot analysis of fractions obtained from homogenates of ACC-pretreated tomato cells by differential centrifugation. *Lane H1*, total homogenate. *Lanes S1, S2, S3* and *P1, P2, P3*, supernatants and pellets, respectively, after differential centrifugations at 100·g, 18500·g, and 140000·g. *M*, markers. P1 consists mainly of cell walls, P2 mainly of large organelles, and P3 of microsomal membranes

This is in contrast to the situation in fruits, where Rombaldi et al. (1994) recently described a predominantly extracellular localization of ACC oxidase.

Vacuoles were prepared and purified from the protoplasts. One-fifth to one-fourth of the α-mannosidase activity initially present in the protoplasts was recovered in the vacuolar fraction, indicating a yield of 20–25%. The purified vacuoles were examined in the light microscope. About 90–95% of the objects counted were clean vacuoles, but there was a contamination of 5–10% protoplasts. Cytoplasmic contamination was also assessed by measuring malate dehydrogenase activity; it had a level of 5–15% in purified vacuoles, as compared with the original protoplasts (Table 1). Protoplast and vacuole preparations were adjusted to equal α-mannosidase levels and then subjected to immunoblot analysis: there was no discernible signal of ACC oxidase in the vacuole preparation (Fig. 3B). The activity of ACC oxidase, measured in vivo in protoplasts and vacuoles, correlated with the cytoplasmic marker malate dehydrogenase (Table 1). These findings clearly demonstrate that ACC oxidase is localized in an extravacuolar compartment in the tomato cells used in this study, and are in contrast to previous studies with leaf protoplasts where up to 80% of ACC oxidase was found to be localized in vacuoles (Guy and Kende 1984; Mayne and Kende 1986; Guy 1990).

To further study a possible membrane association of ACC oxidase, homogenates from cells treated overnight with ACC were subjected to differential centrifugation. Pellets generated at 100·g, 16500·g, and 140000·g did not show any immunoreaction with the antibodies against ACC oxidase, and all the antigen was recovered in the final 140000·g supernatant (Fig. 4). Thus, the ACC oxidase studied here is a soluble cytoplasmic enzyme when expressed in its native environment, and there are no indications for a membrane association, in contrast to the case of its heterologous expression in yeast (Peck et al. 1992). This finding is compatible with the amino-acid sequence of ACC oxidase derived from its cDNA, which is devoid of membrane-association signals (Hamilton et al. 1991; Spanu et al. 1991).

Conclusion. ACC oxidase is a soluble cytoplasmic enzyme in suspension-cultured tomato cells, and there is no evidence for an extracellular or vacuolar localization. It remains to be seen if the enzyme can be exported to the

extracellular space under specific conditions, such as those occurring perhaps during fruit ripening (Rombaldi et al. 1994).

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