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Involvement of calcium in ACC-oxidase activity from *Cicer arietinum* seed embryonic axes

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Abstract

Both in vivo and in vitro ACC-oxidase activities as well as ethylene production from embryonic axes of chickpea seeds were strongly inhibited by EGTA, a selective extracellular Ca^{2+} ion chelator, indicating that the influx of Ca^{2+} is important for enzymatic activity. EGTA inhibition was restored by exogenous Ca^{2+}. Treatments of embryonic axes with either Verapamil and $LaCl_3$ (both Ca^{2+} channel blockers) or TMB-8 (an intracellular Ca^{2+} antagonist) provoked an inhibition of both ACC-oxidase activity and ethylene production. These results suggest an involvement of calcium fluxes and intracellular calcium levels in the activity of the last step of the ethylene biosynthetic pathway, which is, in turn, intimately correlated with germination of *Cicer arietinum* seeds.

Keywords: *Cicer arietinum*; Fabaceae; Chickpea; Embryonic axis; Calcium; Ethylene; ACC-oxidase in vitro and in vivo

1. Introduction

Higher plants utilize Ca^{2+} as a major cellular and physiological regulator (Bush, 1989). Effects of Ca^{2+} on phytohormone action have also been reported (Bethke, Gilroy, & Jones, 1995), suggesting that Ca^{2+} can mediate the action of plant growth substances. Thus, strong evidence implicates Ca^{2+} in certain phytohormone-mediated developmental events, including those involving auxin (Bourboulous, Roblin, & Fleurat-Lessard, 1992; Cho & Hong, 1996), gibberellins (Montague, 1993; Bethke et al., 1995), cytokinins (Conrad & Hepler, 1988), ABA (Bethke et al., 1995; Van der Meulen, Visser, & Wang, 1996) and ethylene (Cheverry, Pouliquen, Le Guyader, & Marcellin, 1988; Sánchez-Calle, Delgado, Bueno, Díaz-Miguel, & Matilla, 1989; Raz & Fluhr, 1992). However, scant data are available on the participation of Ca^{2+} ions and calcium channels in the activity of enzymes involved in phytohormone biosynthesis, specially with respect to ethylene. In higher plants, ethylene is produced by 1-aminocyclopropane-1-carboxylic acid (ACC) oxidation, mediated by ACC-oxidase, a dioxygenase that uses ascorbate instead of α-ketoglutarate as a reductant and has an absolute requirement for CO_2 for activity (Kende, 1993). Ethylene biosynthesis is strictly regulated, and is induced by a number of different signals, including pathogen infection, auxins and developmental factors in senescence of flowers and ripening fruit (Fluhr & Mattoo, 1996). In some of these signals inducing ethylene, Ca^{2+} is involved (Raz & Fluhr, 1992). Here, we provide data concerning the effect of Ca^{2+} on ACC-oxidase from embryonic axes of *Cicer arietinum* seeds. Recently, we demonstrated that ACC-oxidase activity has a preponderant role in this seed, which requires ethylene production to germinate (Muñoz De Rueda, Gallardo, Matilla, & Sánchez-Calle, 1995; Matilla, 1996).

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Table 1
Effect of EGTA on in vivo ACC-oxidase activity and ethylene production of embryonic axes of chickpea seeds

EGTA(mM)	ACC-oxidase[a] (nl g^{-1} FW h^{-1})	Ethylene production in the absence of added ACC (nl g^{-1} FW h^{-1})
None	854 ± 11	85 ± 9
5	785 ± 15	83 ± 3
10	745 ± 21	60 ± 2
20	351 ± 18	43 ± 2
30	159 ± 16	32 ± 3
40	25 ± 15	n.d.[b]

[a]250 mg of embryonic axes which had been incubated for 6 h with increasing concentrations of EGTA were incubated for 2 h in the presence of a saturating concentration of ACC (1 mM) and the ethylene production was then determined over the subsequent 30 min. Results are the mean of 2–3 replicate experiments ± S.D.
[b]n.d. means not detected.

2. Results and discussion

Previously we demonstrated that, in embryonic axes isolated from *C. arietinum* seeds grown for 48 h in an appropriate nutritive solution, ethylene production reached a maximum at 18 h, this production being stimulated in the presence of exogenous $CaCl_2$ (1–10 mM) but inhibited by the addition of EGTA (1–10 mM) (Sánchez-Calle et al., 1989). On the basis of these results, and after biochemically characterizing the ACC-oxidase in embryonic axes of chickpea seeds (Muñoz De Rueda et al., 1995), we now provide new data on the effect of alterations of intra- and extracellular Ca^{2+} on the activity of this enzyme.

The presence of EGTA, a selective Ca^{2+} chelator which reduces free available Ca^{2+} in the extracellular solution (Gilroy & Jones, 1994), decreases ethylene production in the embryonic axes of chickpea seeds. This decline is correlated with the concentration of EGTA, so that at 40 mM of EGTA the ethylene measured was 3% of that evaluated in the absence of apoplastic Ca^{2+} chelator (Table 1). However, the in vivo activity of the ACC-oxidase could not be detected at 40 mM of EGTA. The addition of 30 mM EGTA, which decreased ethylene production by 82%, inhibited in vivo ACC-oxidase by 63%, with respect to the control. However, in vivo ACC-oxidase and ethylene production was completely inhibited when 40 mM EGTA and 2 mM TMB-8 were both present in the germination medium (data not shown). On the other hand, the presence of EGTA in the reaction cocktail of the in vitro ACC-oxidase determination, inhibited the enzymatic activity concomitantly with the EGTA concentration, reaching 94% inhibition with 0.5 mM EGTA (Table 2). For the experiment represented in Table 3, the supernatant contained 500 μM of EGTA in all samples and then the ACC-oxidase activity was assayed on adding increasing quantities of Ca^{2+} to the reaction medium. This experiment showed that Ca^{2+} at the submillimolar concentrations used, reversed the ACC-oxidase activity inhibited by EGTA. In addition, the in vitro inhibition of the ACC-oxidase, caused by a higher EGTA concentration (40 mM), was also reversed by 10 mM Ca^{2+} (data not shown). The effect of EGTA on in vivo ACC-oxidase activity appears to indicate that the presence of a minimal Ca^{2+} level in the apoplastic space is necessary for ACC-oxidase activity. Several reports published describe how cytosolic Ca^{2+} can act as a second messenger in plant cells (Bush, 1989; Gilroy & Jones, 1994; Van der Meulen et al., 1996); however, it has been difficult to determine if the increase of cytosolic Ca^{2+} occurs by an influx from the apoplastic solution into the cytoplasm, or by an efflux from intracellular organells.

Table 2
Effect of EGTA on in vitro ACC-oxidase[a] activity of embryonic axes of chickpea seeds

EGTA (μM)	(nl g^{-1} FW h^{-1})	(nl mg^{-1} protein h^{-1})
None	1.185 ± 21	28 ± 1
5	1.004 ± 18	23 ± 2
25	864 ± 20	20 ± 2
50	672 ± 18	16 ± 3
100	437 ± 37	10 ± 4
500	25 ± 15	2 ± 1

[a]The assay mixture contained the 28,000*g* supernatant fraction of 500 mg embryonic axes. The rates shown are the mean of 2–3 replicate experiments ± S.D.

To analyze the effect of mobilization of intracellular Ca^{2+} in relation to ACC-oxidase activity, we performed a series of experiments with several chemical agents which alter the compartmentalization of this cation. Treatment with Verapamil, which blocks the entry of Ca^{2+} into the cell through gated voltage-dependent calcium channels and thereby suppresses the increase in cytoplasmic Ca^{2+} levels (Lee & Tsein,

Table 3
Effect of EGTA (500 μM) plus increasing Ca^{2+} concentrations on in vitro ACC-oxidase[a] activity of embryonic axes of chickpea seeds

Ca_2Cl (μM)	(nl g^{-1} FW h^{-1})	(nl mg^{-1} protein h^{-1})
None	71 ± 21	2 ± 1
100	65 ± 10	2 ± 1
250	143 ± 16	5 ± 1
500	508 ± 20	18 ± 3
750	579 ± 21	20 ± 2
1000	617 ± 18	22 ± 3
2000	787 ± 22	27 ± 4

[a]The assay mixture contained the 28,000*g* supernatant fraction of 500 mg embryonic axes, EGTA (500 μM) and increasing quantities of Ca^{2+} (as $CaCl_2$). The results are expressed as in Table 2 and are the mean of 2–3 replicate experiments ± S.D.

Table 4
Effect of Verapamil on in vivo ACC-oxidase activity and ethylene production of embryonic axes of chickpea seeds

Verapamil (μM)	ACC-oxidase[a] (nl g^{-1} FW h^{-1})	Ethylene production in the absence of added ACC (nl g^{-1} FW h^{-1})
None	851 ± 9	86 ± 10
5	802 ± 7	74 ± 9
25	665 ± 9	73 ± 8
100	814 ± 8	58 ± 5
500	772 ± 6	42 ± 6
1000	n.d.	n.d.[b]

[a]250 mg embryonic axes which had been incubated for 6 h with increasing concentrations of Verapamil were incubated for 2 h in presence of a saturating concentration of ACC (1 mM) and the ethylene production was then determined over the subsequent 30 min. Results are the mean of 2–3 replicate experiments ± S.D.
[b]n.d. means not detected.

Table 5
Effect of $LaCl_3$ on in vivo ACC-oxidase activity and ethylene production of embryonic axes of chickpea seeds

$LaCl_3$(mM)	ACC-oxidase[a] (nl g^{-1} FW h^{-1})	Ethylene production in the absence of added ACC (nl g^{-1} FW h^{-1})
None	845 ± 11	80 ± 5
0.01	773 ± 10	85 ± 4
0.1	839 ± 15	114 ± 9
1	852 ± 12	109 ± 8
10	574 ± 10	141 ± 9
50	311 ± 14	54 ± 3
100	132 ± 10	n.d.[b]

[a]250 mg of embryonic axes which had been incubated for 6 h with increasing concentrations of $LaCl_3$ were incubated for 2 h in presence of a saturating concentration of ACC (1 mM) and ethylene production was then determined over the subsequent 30 min. Results are the mean of 2–3 replicate experiments ± S.D.
[b]n.d. means not detected.

1983; Harvey, Venis, & Trewavas, 1989), inhibited ethylene production as well as ACC-oxidase activity (Table 4). In fact, at 500 μM it had a very small (15%) effect on ACC-oxidase activity, but a much larger effect (50%) on ethylene production. At 1000 μM, Verapamil completely abolished enzymatic activity and ethylene production, suggesting that the Ca^{2+} traffic crossing the plasma membrane could be involved in this activation process. However, exogenous Ca^{2+} did not reverse the inhibition of Ca^{2+} channels by Verapamil or EGTA plus Verapamil (data not shown), because of the irreversibility of binding of Verapamil to Ca^{2+} channels (Cho & Hong, 1996). A comparable behavior for the Verapamil response in the embryonic axes of chickpea seeds was found in phytoalexin induction in oats (Ishihara, Miyagawa, Kuwahara, Veno, & Mayama, 1996). When $LaCl_3$, another Ca^{2+} channel blocker, was added to the external medium, inhibition was recorded in ethylene production starting at 10 mM $LaCl_3$ and ACC-oxidase activity starting at 50 mM $LaCl_3$ (Table 5). Finally, the Ca^{2+} antagonist TMB-8, which acts by inhibiting intracellular Ca^{2+} movement, appreciably inhibited both ethylene production and in vivo ACC-oxidase activity concomitantly to the increase in submicromolar concentrations of TMB-8 (Table 6). When 500 μM Ca^{2+} was added to the medium containing TMB-8, the in vivo ACC-oxidase was fully reversed. However, Ca^{2+} concentrations ranging from 50 μM to 2 mM were unable to fully reverse ethylene production (data not shown).

An understanding of the results of the present work depends on the knowledge on the cellular localization of the ACC-oxidase activity in the biological system under study. Until now, ACC-oxidase activity has been located in apoplastic (Rombaldi et al., 1994) and cytosolic (Reinhardt, Kende, & Boller, 1994) spaces from tomato fruits. By means of immunogold immunocytochemistry with ACC-oxidase polyclonal antibodies recently raised for us to ACC-oxidase protein from chickpea seeds, we can now confirm the location of ACC-oxidase in both apoplast and cytosol of elongating cells of the embryonic axis. This will be described in another publication.

3. Experimental

3.1. Chemicals

1-Aminocyclopropane-1-carboxylic acid, calcium chloride, lanthanum chloride, ethylene glycol-bis (β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid, 3,4,5,-trimethoxybenzoic acid 8-(diethyl-amino)-octyl ester and (±)-verapamil hydrochloride came from Sigma.

Table 6
Effect of TMB-8 on in vivo ACC-oxidase activity and ethylene production of embryonic axes of chickpea seeds

TMB-8	ACC-oxidase[a] (nl g^{-1} FW h^{-1})	Ethylene production in the absence of added ACC (nl g^{-1} FW h^{-1})
None	860 ± 10	94 ± 8
1	827 ± 8	86 ± 5
10	764 ± 7	70 ± 3
100	–	66 ± 12
500	553 ± 11	54 ± 4
1000	–	38 ± 5
2000	99 ± 15	13 ± 4

[a]250 mg of embryonic axes which had been incubated for 6 h with increasing concentrations of TMB-8 were incubated for 2 h in presence of a saturating concentration of ACC (1 mM) and the ethylene production was then determined over the subsequent 30 min. Results are mean of 2–3 replicate experiments ± S.D.
[b]n.d. means not detected.

3.2. Plant material

C. arietinum L. cv. Castellana seeds, harvested in 1996, were stored dry at 0–4°C until used.

3.3. Seed germination

Seeds were germinated at 25°C in darkness on H_2O-moistened filter paper in sterile plastic trays. The germination time was 12 h and the embryonic axes were isolated aseptically and then incubated for 6 h in Petridishes containing the indicated treatments (Verapamil, EGTA, EDTA, TMB-8, $CaCl_2$ or $LaCl_3$).

3.4. Determination of ethylene

Samples (250 mg) of appropriately 6 h treated embryonic axes were transferred to 20 ml flasks containing 0.25 ml of the various treatment solns. After a 30 min incubation period the atmosphere in the vials was sampled for ethylene determination as described in Gallardo, Sánchez-Calle, Muñoz De Rueda and Matilla (1996).

3.5. In vivo ACC-oxidase activity

Embryonic axes (250 mg) incubated for 6 h with the calcium-related compounds were treated for 2 h at 25°C with 1 mM ACC. After this period, the vials (20 ml) were sealed with silicone rubber stoppers and 30 min later 1 ml samples were withdrawn from flasks and the ethylene concentration measured by FID-GC. Further details were as described in Muñoz De Rueda et al. (1995).

3.6. Extraction and in vitro assay of ACC-oxidase

Samples (500 mg) were homogenized in a mortar with 1 ml of extraction buffer (200 mM HEPES, pH 7.0 and 10 vol% glycerol). The homogenate was centrifuged at 28,000*g* for 20 min and the supernatant was used for the ACC-oxidase assay. The ACC oxidase activity was assayed by GC determination of the ethylene produced after incubation for 1 h at 30°C in capped 12 ml vials containing a reaction mixture (2 ml) composed of: 0.2 M HEPES, pH 7.0, 10% glycerol, 4 μM $FeSO_4$, 6 mM Na-ascorbate, 1 mM ACC, 20% O_2 (in the gas phase) and 30 mM Na-bicarbonate. The reaction was started by the addition of 100 μl of the above supernatant. The controls were reactions with or without boiled enzymatic extract and in neither case was ethylene production detected. The protein concentration was determined following Bradford (1976), using BSA as a standard.

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