Changes of 1-aminocyclopropane-1-carboxylic acid oxidase activity in stressed *Pinus sylvestris* needles

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Abstract

Stimulation of ethylene biosynthesis in pine needles by hydrogen peroxide and sodium bisulfite coincided with the activation of ACC oxidase at the level of protein synthesis. Decrease in ethylene production at high concentrations of sodium bisulfite (above 7 mM) was apparently due to inhibition of ACC oxidase activity. Treatment of pine needles with aminotriazole caused an inhibition of both ethylene production and ACC oxidase activity. Both methylviologen and methyl jasmonate stimulated ACC oxidase activity in a concentration-dependent manner with no parallel changes in ethylene production. The presented results suggest that ACC oxidase plays an important role in regulation of ethylene formation in pine needles in response to different stimuli.

Additional key words: ethylene biosynthesis, pine.

Introduction

Ethylene is an endogenous plant hormone involved in plant responses to a wide array of stress factors. Ethylene together with jasmonates are considered the major signal compounds for wound-induced gene expression in plants (Hiraga *et al.* 2000). Induction of ethylene biosynthesis by stress is a tightly regulated process presumably involving induction of two enzymes of ethylene biosynthesis pathway, namely, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase. In many tissues ACC synthase is believed to be the rate-limiting step in ethylene formation. Thus, a wide variety of chemical factors increase ethylene biosynthesis at the level of steady-state level of ACC synthase mRNA (Avni *et al.* 1994).

Recent results suggest that usually two groups of ACC oxidase transcripts are expressed in vegetative tissues, either stress-induced or constitutive (Kim *et al.* 1998). The growing number of results have demonstrated that the ACC oxidase gene family is highly regulated during plant development as well (Hunter *et al.* 1999).

During studies of putative ACC independent pathway of ethylene biosynthesis in stressed pine (*Pinus sylvestris* L.) needles (Ievinsh and Tillberg 1995) it was established that ethylene is produced in the presence of inhibitors of ACC synthesis in spite of complete inhibition of stress-dependent increase of ACC pool. It was suggested that increase in ACC oxidase activity may count for inhibitor-insensitive component of ethylene production. The present experiments were designed to study the role of ACC oxidase in ethylene biosynthesis in chemically stressed pine needles with a particular emphasis on possible effects of inhibitors of ACC synthesis on ACC oxidase activity.

Materials and methods

One-year-old pine seedlings were obtained from a local nursery during the rest period. Seedlings were transplanted to pots containing soil and placed in a growth cabinet at a photosynthetic photon flux density of 320 μmol m^{-2} s^{-1} during a 12-h photoperiod, day/night temperature of 15/10 °C, and relative humidity 75 ± 10 %. Bud break was induced by prolongation of the day to 14 h and increasing the day temperature to 20 °C. After start

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start of elongation, the light period was shifted to 16 h, and the temperature conditions were changed to 25 °C during the day and 15 °C during the night. Visually non-damaged completely elongated current year needles were used for experiments.

As it was shown that a simple *in vitro* assay of ACC oxidase activity in crude extracts from pine needles can not be used to measure a native ACC oxidase activity (Kruzmane and Ievinsh 1999), in the present experiments *in situ* assay for ACC oxidase was used. Preliminary experiments have showed that 1 mM of ACC caused saturation of ethylene formation in pine needles. When pine needles were incubated in 1 mM ACC, 4 h were necessary for even distribution of internal concentration of ACC in all parts of the needle. Therefore, for all the further experiments, for measurement of ACC oxidase activity, pine needles were incubated in 1 cm^3 of 1 mM ACC for 3 h in an open 4 cm^3 borosilicate glass bottles, then the bottles were closed with teflon coated silicone caps and incubated for 1 h.

For treatments, about 0.5 g of freshly detached needles were placed in 4 cm^3 bottles containing 0.5 cm^3 of the appropriate media. Bottles were kept in the same conditions as used for seedling growth. At the end of the incubation period, needles were withdrawn from the medium and were transferred to 4 cm^3 bottles with 1 mM ACC for determination of ACC oxidase activity. For measurement of ethylene production intensity, needles were incubated in the appropriate media in sealed bottles, concentration of ethylene was determined at the end of the incubation period.

Concentration of ethylene in gas samples was analyzed by a gas chromatograph *Chrom 5* equipped with a glass column filled with activated Al_2O_3 and the flame ionization detector. Helium was used as a carrier gas. Oven temperature was 80 °C, temperature of the detector 110 °C.

Results

Effect of H_2O_2 and $Na_2S_2O_5$ on ACC oxidase activity: To test whether H_2O_2- and $Na_2S_2O_5$-induced changes in ethylene production intensity in pine needles are accompanied by changes of ACC oxidase activity, dose responses of H_2O_2 and $Na_2S_2O_5$ were performed. H_2O_2 concentration of 0.3 M was necessary to saturate the increase in ACC oxidase activity after 12 h of incubation (Fig. 1). In $Na_2S_2O_5$-treated needles, maximum induction of ACC oxidase activity was found at 6 mM (Fig. 2). Further increase of $Na_2S_2O_5$ concentration led to sharp decrease of ACC oxidase activity. For both inducers, ACC oxidase activity increased further in needles incubated for 24 h (data not shown).

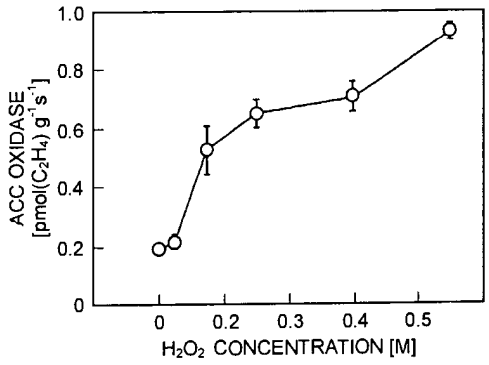

Fig. 1. Dependence of ACC oxidase activity in pine needles on the concentration of H_2O_2 in the incubation medium. Needles were incubated in the appropriate medium for 12 h, then were transferred to 1 mM ACC for 4 h. Produced ethylene was accumulated within the last 1 h of incubation in ACC. Means ± SE, $n = 4$.

Additional treatment of pine needles with cycloheximide, an inhibitor of cytoplasmic protein synthesis, completely inhibited increase in ACC oxidase activity due to H_2O_2 and $Na_2S_2O_5$ (Fig. 3). Cycloheximide strongly inhibited control ACC oxidase activity as well.

Inhibitor of ACC synthase, aminooxyacetic acid, had no effect on ACC oxidase activity in pine needles over a wide range of concentrations (data not shown).

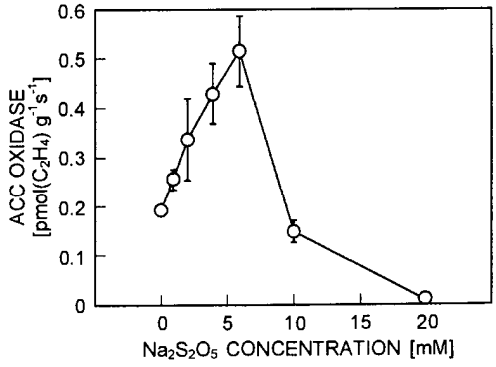

Fig. 2. Dependence of ACC oxidase activity in pine needles on the concentration of $Na_2S_2O_5$ in the incubation medium. Needles were incubated in the appropriate medium for 12 h, then were transferred to 1 mM ACC for 4 h. Produced ethylene was accumulated within the last 1 h of incubation in ACC. Means ± SE, $n = 4$.

Effect of aminotriazole, methylviologen and methyl jasmonate on ethylene production and ACC oxidase activity: Several other potential inhibitors/activators of ethylene biosynthesis were tested in a pine needle system.

Treatment of pine needles with aminotriazole, an inhibitor of catalase activity, caused an inhibition of both ethylene production intensity as well as ACC-oxidase activity (Fig. 4). Identical concentration of aminotriazole (20 mM) was necessary to saturate the response both for ethylene production and ACC oxidase.

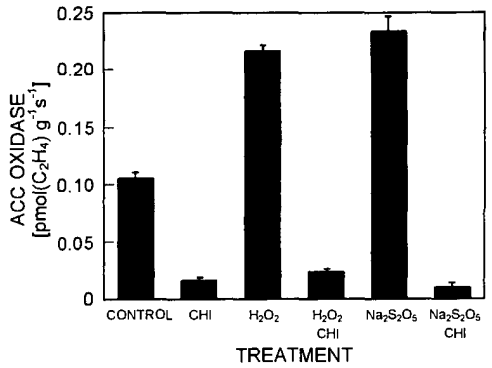

Fig. 3. Effect of cycloheximide (CHI) on stress-induced ACC oxidase activity in pine needles. Needles were incubated in water (control), 0.3 M H_2O_2, or 6 mM $Na_2S_2O_5$ with or without 1 mM cycloheximide for 12 h. After that, they were transferred to 1 mM ACC for 4 h. Produced ethylene was accumulated within the last 1 h of incubation in ACC. Means ± SE, $n = 4$.

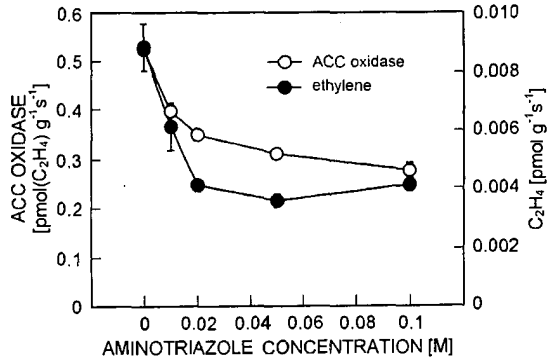

Fig. 4. Effect of different concentrations of aminotryazole on ethylene production intensity and ACC oxidase activity in pine needles. For measurement of ethylene production intensity, needles were incubated in the appropriate media in a stopper-closed 4 cm^3 glass bottles for 24 h. Produced ethylene was measured at the end of incubation period. For measurement of ACC oxidase activity, needles were incubated in the appropriate media for 24 h, then they were transferred to 1 mM ACC for 4 h. Produced ethylene was accumulated within the last 1 h of incubation in ACC. Means ± SE, $n = 4$.

Methylviologen, an inducer of endogenous oxidative stress, widely used as a herbicide, stimulated ACC oxidase activity in pine needles already at 2 µM (Fig. 5). Maximum activation was reached at 10 µM methylviologen in the incubation medium. Curiously, there was no effect of methylviologen on ethylene production up to concentration of 50 µM. However, methylviologen-dependent increase of ACC oxidase activity was partially inhibited by cycloheximide (data not shown).

Wound-signal compound methyl jasmonate caused an increase of ACC oxidase activity in pine needles (Fig. 6). Methyl jasmonate at concentration of 5 µM saturated the ACC oxidase response. In contrast, a considerable increase of ethylene production intensity from methyl jasmonate-treated pine needles was monitored only at 20 µM (Fig. 6).

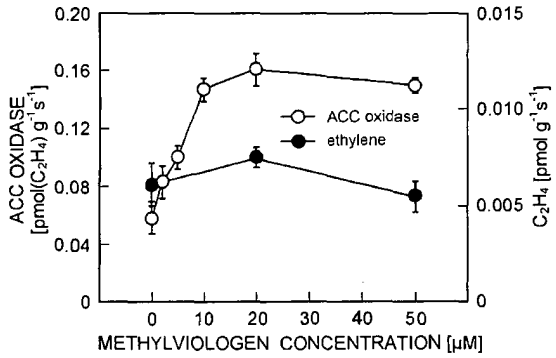

Fig. 5. Dependence of ethylene production intensity and ACC oxidase activity in pine needles on the concentration of methylviologen in the incubation medium. For measurement of ethylene production intensity, needles were incubated in the appropriate media in a stopper-closed 4 cm^3 glass bottles for 24 h. Produced ethylene was measured at the end of incubation period. For measurement of ACC oxidase activity, needles were incubated in the appropriate media for 24 h, then they were transferred to 1 mM ACC for 4 h. Produced ethylene was accumulated within the last 1 h of incubation in ACC. Means ± SE, $n = 4$.

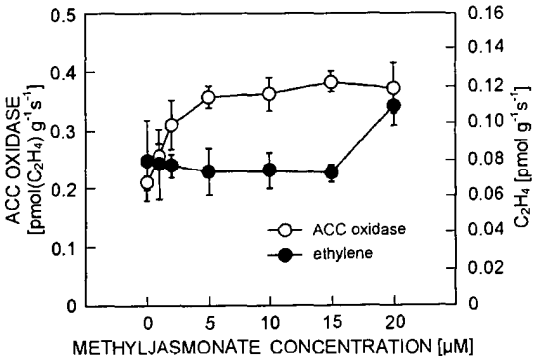

Fig. 6. Dependence of ethylene production intensity and ACC oxidase activity in pine needles on the concentration of methyl jasmonate in the incubation medium. For measurement of ethylene production intensity, needles were incubated in the appropriate media in a stopper-closed 4 cm^3 glass bottles for 12 h. Produced ethylene was measured at the end of incubation period. For measurement of ACC oxidase activity, needles were incubated in the appropriate media for 12 h, then they were transferred to 1 mM ACC for 4 h. Produced ethylene was accumulated within the last 1 h of incubation in ACC. Means ± SE, $n = 4$.

Discussion

Activation of ethylene biosynthesis can be observed at the level of both ACC synthesis and ACC oxidase in response to various stress factors, *e.g.*, H_2O_2 and $Na_2S_2O_5$. ACC oxidase activation is most probably due to *de novo* protein synthesis. In pine needles treated with H_2O_2 and $Na_2S_2O_5$ for 24 h, there is a decrease of ethylene production in spite of continuously growing levels of ACC and increasing ACC oxidase activity. The phenomenon might be related to an increase of ACC conjugation or due to the transport of ACC to compartments not accessed by ACC oxidase.

Ethylene production decreased in parallel with decrease in ACC oxidase activity, *e.g.*, in pine needles treated with high concentrations of $Na_2S_2O_5$ (above 7 mM) or with aminotriazole. Aminotriazole is known to inhibit ethylene formation at the level of ACC synthesis without any direct effect on the enzyme activity *in vitro* (Altman and Solomos 1994). The exact mechanism by which aminotriazole affects ethylene biosynthesis is not known. In pine needles, aminotriazole is an effective inhibitor of catalase, causing 10-fold decrease of the minimal concentration of H_2O_2 necessary for induction of antioxidative responses (Ievinsh *et al.* 1995).

Increase of ACC oxidase activity was dose-dependent with no concomitant significant changes in ethylene production in methylviologen and methyl jasmonate treated needles. In *Nicotiana glutinosa*, methyl jasmonate was shown to increase transcript levels of ACC oxidase (Kim et al. 1998). Several reports exist on concentration-dependent inhibitory effect of methyl jasmonate on wound-inducible ACC oxidase expression as well (Kim and Yang 1994). Only extremely limited information is available on the effect of methylviologen on ethylene biosynthesis. It was shown that methylviologen stimulates ACC synthesis in *Phaseolus vulgaris* seedlings (Weckx *et al.* 1989).

Decrease of ACC oxidase activity in cycloheximide- or cycloheximide plus $Na_2S_2O_5$-treated needles was in parallel with increase of ethylene production intensity. As aminooxyacetic acid caused a partial inhibition of both cycloheximide and cycloheximide plus $Na_2S_2O_5$ induced ethylene production (Ievinsh and Tillberg 1995), it is possible that the activation was due to increase of ACC synthesis. As cycloheximide completely inhibited both H_2O_2-induced as well as $Na_2S_2O_5$-induced increase of ACC oxidase activity, the stimulatory effect of cycloheximide on ethylene production from $Na_2S_2O_5$-treated pine needles described in previous experiments (Ievinsh and Tillberg 1995) is not associated with increase in ACC oxidase activity. Our data are different from those reported recently that substantial accumulation of ACC oxidase transcripts occurs in melon tissues treated with cycloheximide (Bouquin *et al.* 1997). There it was proposed that ACC oxidase gene is regulated by either a short-lived repressor protein or by a rapid turn-over of a RNAse responsible for degradation of transcripts.

Inhibitor of ACC synthase, aminooxyacetic acid, had no effect on ACC oxidase activity in pine needles over a wide range of concentrations. It was proposed earlier that the increase of ACC oxidase activity in stressed pine needles is the simplest explanation for the incerase of ethylene production in the presence of inhibitors (*e.g.*, aminooxyacetic acid) in spite of the unchanged ACC pool (Ievinsh and Tillberg 1995). Therefore, the existence of inhibitor-insensitive ethylene production in the pine needle system (Ievinsh and Tillberg 1995) could not be due to stimulatory effect of inhibitors on ACC oxidase activity.

The important question to answer is whether ACC oxidase may be a rate limiting step in ethylene biosynthesis in vegetative tissues. The presented results are consistent with the suggestion that ACC oxidase plays an important role in regulation of ethylene formation in vegetative plant tissues in response to different stimuli. Now it is necessary to identify the endogenous signals specifically regulating the expression of ACC oxidase.

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