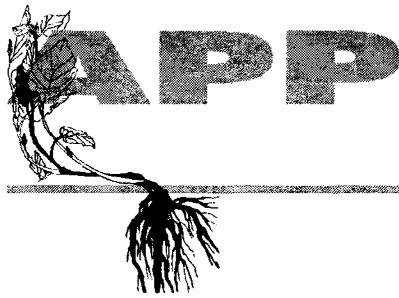

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The *in vivo* and *in vitro* influence of methyl jasmonate on oxidative processes in *Arabidopsis thaliana* leaves

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Key words: *Arabidopsis*, ascorbate, hydrogen peroxide, jasmonate, stress, superoxide dismutase

Abstract

In *Arabidopsis thaliana* leaves a strong increase of H_2O_2 content was induced by application of methyl jasmonate (JAMe) through the root system, but the induction only slightly depended on JAMe concentration. The activity of superoxide dismutase and ascorbic acid peroxidase increased at lower JAMe concentrations and decreased at higher ones. Catalase activity decreased proportionally to JAMe concentration (in comparison with control plants). The sum of ascorbic acid and dehydroascorbate content at 10^{-6} M JAMe was similar to the control, but at higher concentrations it increased, especially due to a higher ascorbate accumulation. Methyl jasmonate applied directly to the extract of leaves (*in vitro* experiment) also induced a strong increase in H_2O_2 level, even at a low concentration (10^{-8} M). Since lower JAMe concentrations induced weak superoxide dismutase and did not change catalase and peroxidase activity, it is suggested that in this case a high level of hydrogen peroxide was not the result of the activity of the mentioned enzymes. JAMe-induction of H_2O_2 increase at the highest JAMe concentration resulted from SOD activity. Our *in vivo* and *in vitro* experiments suggest that jasmonate can influence oxidative stress not only through gene expression but also by its direct effect on enzyme activity.

Introduction

Jasmonate molecules are one of the indispensable components of signalling pathways in stressed plants. Their role in regulating many defense genes encoding, for example, proteinase inhibitors, pathogenesis-related and antifungal proteins (Sembdner and Parthier 1993, Penninckx *et al.* 1996, Wasternack and Parthier 1997, León *et al.* 2001) and also some growth and senescence processes (Ueda and Kato 1980, Creelman and Mullet 1997, Saniewski *et al.* 1999) is partially known, but at present there is only minor information concerning the direct influence of jasmonates on metabolic processes in stressed plants. Some of them report jasmonate influence on: H_2O_2 productivity (Orozco-Cardenas and Ryan 1999, Orozco-Cardenas *et al.* 2001), activity of lipoxygenase (Vèronèsi *et al.* 1999), ATP-ase (Ratajczak *et al.* 1998) and polyphenol oxidase (Czapski *et al.* 1988).

Some recent results indicate that between oligosaccharides, ethylene and jasmonates there exists a specific cross-talk regulating locally and systemically synthesis of different proteins in wounded plants (Titarienko *et al.* 1997, Penninckx *et al.* 1998, see also Bostock 1999, Møller and Chua 1999, Rojo *et al.* 1999, Shoji *et al.* 2000). Previously these connections were also found between jasmonates and salicylic acid (Peña-Cortés *et al.* 1993). Signal molecules may function coordinately

with active oxygen species (AOS). These species, especially H_2O_2, play a dual role in defense: a direct antibiotic activity (Peng and Kúc 1992) and indirect effects as mediator of the activation of the other defense components, such as: oxidative cross-linking of cell walls (Ogawa *et al.* 1997) and the expression of genes near the infection site (see Lamb and Dixon 1997). The results obtained by Watanabe *et al.* (2001) provided evidence that timing of AOS, jasmonic acid (JA) and ethylene production influence the plant response to wound stress. They were in agreement with earlier investigations concerning AOS and signal molecule influence on stress-induced apoptosis (see Jabs 1999). Although the rapid increase of H_2O_2 after exposure of plants to many stress factors has been confirmed, the mechanism of its increase is not resolved.

Chloroplasts are unique organelles that contain a relatively high level of unsaturated fatty acids in its membranes. These fatty acids are indispensable components of thylakoids on the one hand, but on the other also in octadecanoic pathways localized mostly in chloroplasts (Bell *et al.* 1995). Therefore, the structure and function of the photosynthetic apparatus can be strongly connected with the level of signal molecules (especially jasmonates) synthesized there. Recently some investigations indicated that octadecanoid pathways might be connected with generation of some components of oxidative burst (Orozco-Cardenas and Ryan 1999, Rao *et al.* 2000). Because the effect of different stress factors is connected with the oxidative response, we studied the oxidative system components, *e.g.* the activity of catalase (CAT), ascorbate peroxidase (Apx), superoxide dismutase (SOD) and the concentration of H_2O_2 and ascorbate (AA) after external treatment with methyl jasmonate (JAMe).

Material and Methods

Seeds of *Arabidopsis thaliana*, after 4 weeks of germination were cultivated hydroponically in full strength modified Hoagland nutrient solution (0.25 mM Fe^{3+} citrate was added). Plants were grown at 23/18 °C day/night temperature and PPFD 140 $\mu mol \cdot m^{-2} \cdot s^{-1}$ under 11/13 h day/night regime. After 6 days (time sufficient to minimalize the stress factors eventually appearing in changed nutrient medium) plants were transferred to a fresh solution containing 10^{-6}, 10^{-5} and 10^{-4} M JAMe. JAMe was finally dissolved in 0.5 ml ethanol (the same amounts of ethanol were introduced to control plants). After next 6 days the leaves were harvested, frozen in liquid nitrogen and kept at -80 °C for all subsequent analyses.

For *in vitro* analyses JAMe (dissolved in ethanol) was introduced at different concentrations to the enzyme extract 5 min before starting reaction (the ethanol content in control and JAMe-treated enzyme extract was 1 µl). To measure the influence of JAMe on AA oxidase (EC 1.10.3.3) from *Cucurbita* (Sigma), a mixture (1 ml) contain 0.89 ml of 0.1 M sodium phosphate buffer (pH 5.6), 100 µM AA, 0.05 units of AA oxidase, and different concentrations of JAMe was used. The AA oxidase activity was determined from decreasing absorbance at 265 nm during 60 s.

All samples were prepared for soluble protein, CAT, Apx and SOD analyses by rapid homogenizing the frozen leaf material in a solution (4 ml/g fresh weight) containing 50 mM Na_2HPO_4/ /KH_2PO_4 (pH 7.0), 0.8 % (v/v) Triton X-100 and 1 % (v/v) PVP. The homogenate was centrifuged at 16,000 x g for 17 min. and the enzyme activities and H_2O_2 content in the supernatant were determined immediately. All operations were performed at 4 °C.

Protein was assayed according to Bradford (1976) using bovine serum albumin for calibration.

Catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) activity was determined spectrophotometrically by monitoring the absorbance changes caused by disappearing H_2O_2 at 240 nm, according to Aebi (1984) in 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM H_2O_2 and enzyme extract.

Ascorbate peroxidase (L-Ascorbate:H_2O_2 oxidoreductase, EC 1.11.1.11) was monitored from the absorbance decrease of the substrate at 290 nm after Nakano and Asada (1981). The reaction mixture contained 25 mM potassium phosphate (pH 7.0), 0.5 mM ascorbate, 0.2 mM H_2O_2, 0.1 mM EDTA and enzyme extract.

Superoxide dismutase (EC 1.1.5.1.1) activity was assayed according to the method of Beauchamp and Fridovich (1971).

The content of AA and DHA was determined according to the modified procedure used by Foyer *et al.* (1983). The frozen leaves (1 g) were ground rapidly with 4 ml ice-cold 2.5 M $HClO_4$. The mixture was stored on ice for 30 min and then neutralized to pH 5.6 with 1.25 mM K_2CO_3. The insoluble debris was removed through centrifugation and the supernatant was used for analysis of ascorbates. When the levels of AA were determined from a decrease of ascorbate absorbance at 265 nm to a new lower stable level, we used a reaction mixture (1 ml) containing 0.89 ml of 0.1 M sodium phosphate buffer (pH 5.6), 0.5 unit of AA oxidase (EC 1.10.3.3) from *Cucurbita* species and 100 µl of extracts. The levels of DHA were determined by DHA reduction to AA by 2 mM DTT. The AA absorption coefficient was 15 $mM^{-1}cm^{-1}$.

H_2O_2 content was determined according to the method of Pick (1986) in a 2-fold concentrated extract of leaves used for analysis of enzyme activities. The reaction mixture (1 ml) contained 50 mM potassium phosphate buffer (pH 7.0), 0.6 M phenol sulfophthalein (phenol red) sodium salt (Sigma), 20 µl horseradish peroxidase (stock solution contained 2000 units/ml, Sigma) and 100 µl extract. After 10 min of incubation at 37 °C, 450 µl of the mixture were centrifuged and the reaction in the supernatant (400 µl) was interrupted by adding 1 ml of 1 M NaOH. The H_2O_2 content was measured by absorbance increase at 600 nm.

The data are the means of four measurements of three independent series ± S.E.

Results and Discussion

Jasmonates, although they are molecules increasing plant resistance to stress factors, can also enhance senescence processes in excised leaves (Ueda and Kato 1980). In our preliminary studies we observed symptoms of toxicity also in intact leaves after JAMe treatment (Maksymiec and Krupa 2002). After 6 days of exposition to 10^{-4} M of JAMe, the Chl $a+b$ content was diminished to 56 % of control level. This effect may be the result of the development of oxidative stress usually appearing after wounding, extreme temperatures or UV irradiation (Doke 1997).

In *Arabidopsis thaliana* leaves the total ascorbate content increased after a higher JAMe treatment, mainly as the result of AA increase (Table). Ascorbate was shown to be differentially induced in response to the stress factors depending also on the plant species and type of plant-pathogen interaction (Gönner and Schlösser 1993, Cuypers et al. 2000, Kuźniak and Skłodowska 2001). Because JAMe significantly influenced the ascorbate status, it is likely that the difference in AA contents may also result from the signalling pathway function. DHA concentration was not changed at 10^{-6} and 10^{-5} M but at 10^{-4} M JAMe it significantly increased to 177 % of control level. Although this effect was observed at a high concentration of JAMe which itself might act as a stress, it is quite possible that a strong jasmonate increase can take place locally in cells and tissues. DHA accumulation after jasmonate treatment could inhibit the photosynthetic machinery (Gara and Tomasi 1999).

To eliminate a possible influence of JAMe on the procedure of AA analysis, a direct effect of JAMe on ascorbate oxidase activity from *Cucurbita* (used in this method) was investigated. At low concentra-

Table. Ascorbate (AA) and dehydroascorbate (DHA) contents in leaves of *Arabidopsis thaliana* treated with different JAMe concentrations. The data are the means of four measurements of three independent series ± S.E.

	JAMe concentrations (M)			
	0	10^{-6}	10^{-5}	10^{-4}
AA (µmol/g FW)	1.65±0.12 (100)	1.65±0.11 (100)	2.74±0.10 (166)	3.89±0.21 (236)
DHA (µmol/g FW)	2.07±0.10 (100)	1.99±0.08 (96)	1.97±0.04 (95)	3.67±0.32 (177)
AA+DHA (µmol/g FW)	3.72±0.12 (100)	3.64±0.09 (98)	4.71±0.09 (127)	7.56±0.24 (203)

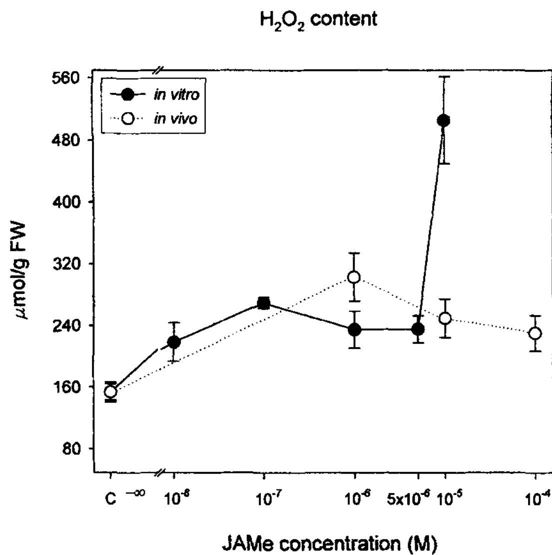

Fig. 1. The *in vivo* and *in vitro* effects of JAMe on H_2O_2 content in leaf extract of *Arabidopsis thaliana* plants. In *in vivo* experiments JAMe was applied through the root system, and *in vitro* directly to the leaf extract of control plants. The data are the means of four measurements of three independent series ± S.E.

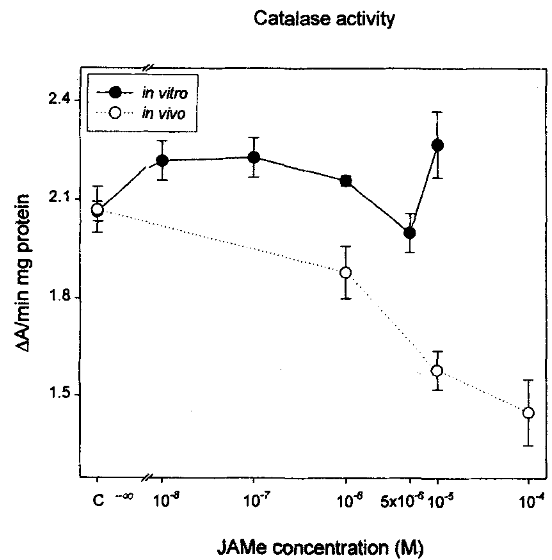

Fig. 2. Catalase (CAT) activity in leaves of control and JAMe-treated *Arabidopsis* plants. The data are the means of four measurements of three independent series ± S.E. (For details see Fig. 1.)

tions (from 10^{-8} to 10^{-6} M) JAMe did not change the enzyme activity. At high concentrations (from 5×10^{-6} to 10^{-4} M) it increased to about 113 – 120 % of control level (data not presented). This result indicated that JAMe did not interfere in the procedure of AA determination if the measurement was performed until the whole pool of AA was oxidized both in control and treated plants.

As shown in Fig. 1 the control leaves of *Arabidopsis thaliana* plants had a relatively high level of H_2O_2 in comparison with other plant species (Biemelt *et al.* 2000, Depège *et al.* 2000). External JAMe, applied through the root system, induced a strong increase in its content. A similar result was observed in the case of direct application of jasmonate to leaf extract and H_2O_2 production corresponded well with JAMe concentration. A rapid H_2O_2 increase is usually connected with the appearance of oxidative burst following the influence of different stress factors on plants (Chain and Doke 1987, Arnott and Murphy 1991, Auh and Murphy 1995). In the present paper, JAMe at a low concentration (10^{-8} M in *in vitro* conditions) increased H_2O_2 content, therefore, it can function as stress factor or signal transmitting molecules, as in copper stress (Xiang and Oliver 1998). Also in tomato leaves methyl jasmonate induced H_2O_2 accumulation (Orozco-Cárdenas and Ryan 1999, Orozco-Cárdenas *et al.* 2001).

H_2O_2 increase may be related to diminution of CAT and Apx activity, or SOD increase, or radical processes. Figure 2 shows that JAMe did not directly inhibit CAT activity. At 10^{-5} M JAMe increased the enzyme activity even to about 110 % of control. However, in *in vivo* conditions CAT activity decreased gradually with JAMe concentration increase. Because H_2O_2 content in leaves did not increase proportionally to JAMe concentration (see Fig. 1), it can be concluded that decreased activity of CAT was not sufficient to induce of H_2O_2 accumulation. Decrease of CAT activity appears to be one of the symptoms of stress damage (Kar *et al.* 1993, Streb *et al.* 1993). However, CAT activity seems to be unaffected by the particular stress factors (Weckx *et al.* 1997, Orendi *et al.* 2000). Jasmonate mediation in stress perception of plants followed by CAT decrease appears to take place only during a longer release of the signal molecule.

Hydrogen peroxide burst was not the result of Apx inhibition because its activity *in vitro* was similar to that of control at three lower JAMe concentrations and increased to about 136 % of control at the highest JAMe concentration (Fig. 3). In *in vivo* condi-

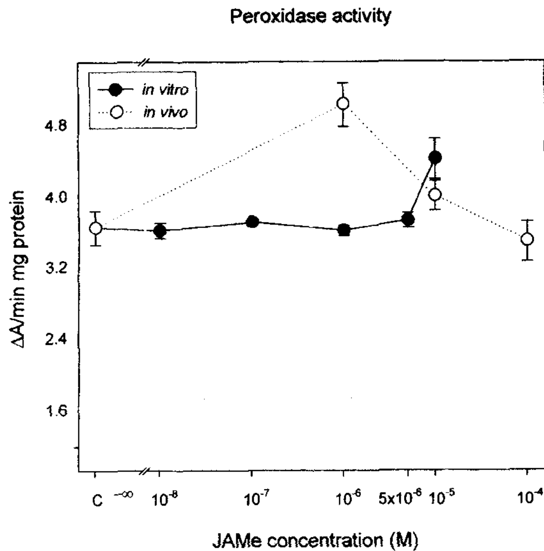

Fig. 3. Ascorbate peroxidase (Apx) activity in leaves of control and JAMe-treated *Arabidopsis* plants. The data are the means of four measurements of three independent series ± S.E. (For details see Fig. 1.)

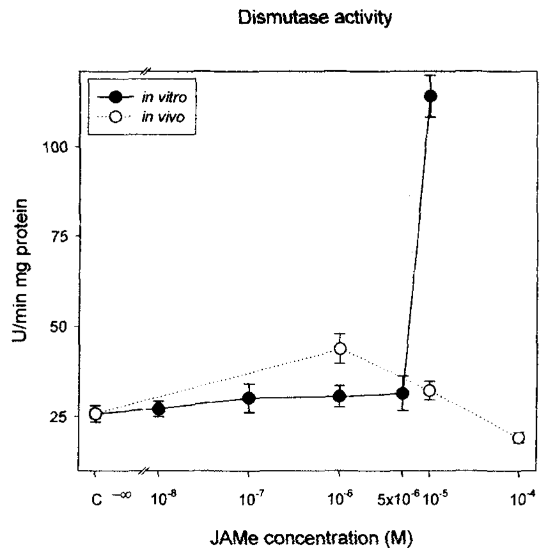

Fig. 4. Superoxide dismutase (SOD) activity in leaves of control and JAMe-treated *Arabidopsis* plants. The data are the means of four measurements of three independent series ± S.E. (For details see Fig. 1.)

tions Apx increased only at 10^{-6} M JAMe concentration, but was it similar or lower at higher concentrations as compared with control.

As seen in Fig. 4, at the highest concentration JAMe strongly increased SOD activity in *in vitro* conditions. A similar effect was observed in the case of H_2O_2. Therefore, we conclude that JAMe-induction of H_2O_2 increase, at the highest JAMe concentration, came from SOD activity. JAMe increased H_2O_2 content also at their lower concentrations. Because in this case SOD activity did not significantly increase, and CAT decrease was parallel to Apx increase we supposed that also other factors could cause hydrogen peroxide burst. Lower JAMe concentrations may activate NADPH oxidase which is probably also responsible for a rapid H_2O_2 generation when stress factors or some signaling molecules are acting (Orozco-Cardenas *et al.* 2001). However, further investigations are needed in this respect. The dynamics of SOD activity after *in vivo* JAMe treatment was similar to the observed changes in H_2O_2 content. SOD activity and H_2O_2 concentration were the highest at 10^{-6} M JAMe and decreased at higher JAMe concentrations. Because in this case CAT and Apx activity also decreased we observed a lower H_2O_2 decrease than SOD. So at the highest JAMe concentration the H_2O_2 level was about 150 % higher than in control, while SOD activity was a little lower than control. It is not excluded that increased AA content after treatment withJAMe at its higher concentration can partially scavenge $O_2^{\cdot-}$ - SOD substrate. In this way SOD activity could be depressing.

It is interesting that in *in vitro* conditions 10^{-5} M JAMe increased activity of the three investigated enzymes (CAT, Apx and SOD to 110, 126 and 442 % of control, respectively). This direct stimulatory effect of JAMe is a new phenomenon observed by us in *Arabidopsis* plants. Because H_2O_2 is a substrate for CAT and Apx we presume that increased CAT and Apx activity may be partially the result of hydrogen peroxide burst to 330 % of control level. A rapid generation of H_2O_2 was also observed in tomato plants within three min. after mechanical treatment connected with the jasmonate pathway (Creelman *et al.* 1992, Depège *et al.* 2000). In *in vivo* conditions, where H_2O_2 did not increase at higher JAMe level, CAT and Apx activity decreased gradually supporting the above conclusion and indicating that jasmonate probably does not directly induce this enzyme activity. Further investigations are needed to resolve this problem.

A rapid increase in SOD activity after direct 10^{-5} M JAMe treatment could be the result of $O_2^{\cdot-}$ generation usually observed in elicitors stimulated cells

(Auh and Murphy 1995). In *in vivo* conditions a high level of AA, observed at 10^{-5} - 10^{-4} M JAMe, is used to scavenge O_2^-, which in consequence decreases SOD activity. Because at a higher H_2O_2 level (obtained after JAMe supply) the activity of this enzyme decreased (Lin and Kao 1998) and the time jasmonate exposition was too short to induce this level of SOD activity, we postulate that JAMe could also modify the enzyme structure.

Increased activity of the above mentioned enzymes at a low (*in vivo* experiment) and a high (*in vitro*) JAME concentration, and their activity diminution at prolonged exposure to its higher concentration could indicate that jasmonate can stimulate the antioxidative defense system only than work at its low level or a short time of action. After a longer time JAMe can depress this system.

In conclusion, our results indicate that JAMe after a long and short exposition affects enzymes of the cellular antioxidative system, ascorbate and especially H_2O_2. The formation of H_2O_2 was largely the result of a rapid increase in SOD activity, but other factors require further investigations. From our *in vivo* and *in vitro* experiments we postulate that jasmonates can directly influence oxidative processes in *Arabidopsis* plants and modify their response to stress factors.

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