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Hydrogen peroxide generated via the octadecanoid pathway is neither necessary nor sufficient for methyl jasmonate-induced hypersensitive cell death in woody plants

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Abstract

Exogenously applied methyl jasmonate (MeJA) might induce the formation of necrotic lesions that closely resemble hypersensitive response lesions. Cellular damage, restricted to the infiltrated zone, was accompanied with the production of H_2O_2 from the oxidative burst. H_2O_2 generated in response to MeJA can be histochemically detected in cells surrounding the necrotic lesions as well as in the vascular tissues. The response is systemic and maximizes with time. Among 12 plant species from different families that were assayed for both hypersensitive reaction (HR)-like response and H_2O_2 generation, only woody species exhibited both MeJA-inducible HR cell death and the generation of H_2O_2. To assess the role of H_2O_2 in MeJA-induced HR-like cell death, a gain and loss of function strategy was employed. The cumulative results indicate that H_2O_2 is neither necessary nor sufficient for MeJA-inducible cell death and that O_2^- rather than H_2O_2 might be responsible.

Additional key words: hypersensitive reaction, jasmonates, programmed cell death, reactive oxygen intermediates.

Introduction

Jasmonic acid (JA) and its methyl ester (methyl jasmonate, MeJA), collectivelly named "jasmonates", act as stress hormones and play a role in plant growth and development (Parthier 1990, 1991, Creelman and Mullet 1997). Previous studies have shown that jasmonates, when applied directly to plants can produce various responses, including growth inhibition, tendril coiling (Creelman and Mullet 1997), and induction of vegetative storage proteins (VSPs) in monocotyledonous and dicotyledonous plants (Muller-Uri *et al.* 1988, Staswick 1989). Jasmonate's ability to cause chlorosis led to the suggestion that this compound plays a role as promotor of plant senescence probably via the chlorophyllase-dependent chlorophyll degradation (Tsuchiya *et al.* 1999).

Several lines of evidence support conclusion that jasmonate plays an important role also in plant insect and disease resistance. First, JA accumulates in wounded plants (Creelman *et al.* 1992) and in plants or cell cultures treated with elicitors of pathogen defense (Gundlach *et al.* 1992). Second, JA activates a diverse arrays of genes encoding protease inhibitors (Johnson *et al.* 1989), antifungal proteins (Penninckx *et al.* 1996, Xu *et al.* 1994), and the ribosome-inactivating protein RIP60 (Chaudry *et al.* 1994). Third, conditioning experiments indicated that plant cells sensitized by the exposure to MeJA respond more intensely and rapidly towards secondary elicitation by fungal pathogen-derived elicitors (Kauss *et al.* 1994, Dubery *et al.* 2000). Results also suggest that JA is involved in or induces a systemic signal for induced resistance as a secondary defense strategy, but clear evidence for an induction of the overall phenomenon of pathogen resistance by MeJA is lacking (Cohen *et al.* 1993).

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Abbreviations: ATZ - 3-amino-1,2,4-triazole; CAT - catalase; DDC - diethyldithiocarbamate; DMSO - dimethylsulfoxide; DPI - diphenyleneiodonium; HPLA - hydroperoxy linolenic acid; HR - hypersensitive reaction; HRP - horse radish peroxidase; NaN_3 - sodium azide; PCD - programmed cell death; PDA - phytodienoic acid; PRX - peroxidase; ROS - reactive oxygen species; SHAM - salicylhydroxamic acid; SOD - superoxide dismutase.

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Recent evidence shows that exogenous MeJA induced a hypersensitive-like response in grapevine leaves that mimic a typical HR symptoms, especially in response to a necrotrophic fungal pathogens (Repka *et al.* 2001). Localized activation of programmed cell death (PCD) that occurs during the HR has been associated with an oxidative burst resulting from the production of reactive oxygen species (ROS) (Mehdy 1994, Baker and Orlandi 1995, Hammond-Kosack and Jones 1996). It is known that the generation of ROS, particularly the perhydroxyl/superoxide radical acid-base pair (HO_2 /O_2^-) and its stable dismutation product, H_2O_2, may be a key mediators of PCD during HR (Levine *et al.* 1994, Jabs *et al.* 1996, Alvarez *et al.* 1998). In addition, nitric oxide (NO), a key signal molecule in animal cells, has also been shown to accumulate during HR formation (for review see Grant and Loake 2000).

H_2O_2, the most stable of the ROS, has been implicated in the cross-linking of different cell wall proteins (Bradley *et al.* 1992), in signal transduction cascade as a regulator of pathogenesis-related (PR-1) gene expression (Chen *et al.* 1995, Chamnongpol *et al.* 1998), in the direct killing of invading pathogens (Peng and Kuc 1992), and in the plant cell death process (Levine *et al.* 1994, Draper 1997). In a recent report, H_2O_2 generated after treatment of tomato leaves with oligosaccharide elicitors (OGA) from plant acts as a second messenger for the induction of defense genes in response to wounding, systemin, and MeJA (Orozco-Cárdenas *et al.* 2001). However, other reports did not implicate H_2O_2 as a key component in phytoalexin synthesis (Jabs *et al.* 1997), or as an initiator of PCD (Devlin and Gustine 1992, Glazener *et al.* 1996, Dorey *et al.* 1999). Furthermore, the "loss and gain-of-function" experiments (Jabs *et al.* 1997) and works with mutants of *Arabidopsis thaliana* (Overmyer *et al.* 2000) demonstrate that O_2^- rather then H_2O_2 is an essential element of the defense-related cell death.

In this study, the role of H_2O_2 generated from the oxidative burst in MeJA-induced HR cell death in the absence of avirulent pathogens was assessed. In addition, it was demonstrated that, unlike to herbaceous plants, woody plant species have a highly sensitive and selective perception system for methyl jasmonate.

Materials and methods

Plants: Barley (*Hordeum vulgare; Poaceae*), cucumber (*Cucumis sativus; Cucurbitaceae*), tobacco (*Nicotiana tabacum; Solanaceae*), bean (*Phaseolus vulgaris; Fabaceae*), aglaonema (*Aglaonema modestum; Araceae*), peperomia (*Peperomia serpens; Peperomiaceae*), oak (*Quercus petraea; Fagaceae*), lime-tree (*Tilia platyphyllos; Tiliaceae*), horse-chestnut (*Aesculus hippocastanum; Aesculaceae*), catalpa (*Catalpa bignoioides; Bignoniaceae*), maple (*Acer pseudoplatanus; Aceraceae*), grapevine (*Vitis vinifera; Vitaceae*), and hibiscus (*Hibiscus rosa-chinensis; Malvaceae*) were grown either from seeds in peat pots or taken as a stem cuttings from mature trees grown in *CRIVE* campus. Plants and cuttings were maintained at 25 ± 1 °C under 17-h photoperiod (30 μmol m^{-2} s^{-1}).

Treatment of plants with MeJA: A stock solution of MeJA (5 mM, *Duchefa*, Haarlem, The Netherlands) was first made up in absolute ethanol; various concentrations of MeJA (0.05, 0.5, 5, and 50 μM) were prepared by appropriate dilution in water and adjusted to the final concentration with 0.1 % ethanol. MeJA was applied at the concentrations indicated as 0.01 cm^3 droplets on leaves (three drops per leaf). Alternatively, plant cuttings in 2.5 cm^3 of water were exposed to MeJA vapour in air-tight *Magenta* containers (*Magenta Corp.*, Chicago, USA) containing cotton-tipped wooden dowels to which had been applied 0.01 cm^3 of dilutions of MeJA in 0.1 % ethanol or 0.1 % ethanol alone as a control. The cotton tip was placed *ca.* 4 cm from the plant leaves. The chambers were incubated at constant irradiance of 30 μmol m^{-2} s^{-1} and temperature of 25 °C for 8 h.

Determination of reactive oxygen species (ROS): Frozen leaves (0.5 g) were homogenized with 1 cm^3 of 0.2 M $HClO_4$ in a precooled minipestle and minimortar (*Kontes*, Vineland, USA). The extract was held on ice for 5 min and centrifuged at 10 000 *g* for 10 min at 4 °C. The supernatant was collected and either processed immediately or quick-frozen at -80 °C until further analysis. All analysis was completed within 72 h of extraction, a period in which no substantial autooxidation of H_2O_2 was observed. The acidic supernatant was neutralized to pH 7.0 to 8.0 with 0.2 M NH_4OH (pH 9.5) and briefly centrifuged at 3 000 *g* for 2 min to sediment the insoluble material. The coloured components in the extract were removed by applying the extract (0.5 cm^3) to a 2-cm^3 column of *AG IX-8* resin (*Bio-Rad*, Hercules, USA) and eluting with 3 cm^3 of deionised water. Generation of H_2O_2 was monitored by chemi-luminescence from the ferricyanide-catalyzed oxidation of luminol (*Sigma*, Deisenhofen, Germany) as described by Schwacke and Hager (1992). The luminescence was detected over a 30-s period with a luminometer (model *FB12*, *Berthold*, Pforzheim, Germany). H_2O_2 concentrations were obtained by calibrating the counts to a standard curve generated with known amounts of H_2O_2 treated essentially as described above.

For measurement of O_2^- generation cytochrome *c* reduction was followed according to the method

described by Doke (1985), using 0.05 cm^3 homogenate in a final volume of 1 cm^3.

***In vivo* detection of H_2O_2 in plants and excised leaves:** H_2O_2 was histochemically detected in the leaves of plants by using 3,3′-diaminobenzidine (DAB) as substrate (Thordal-Christensen *et al.* 1997). Briefly, plants were excised at the base of leaves or at the base of stems with a razor blade and supplied through the cut petioles or stems with a 1 mg cm^{-3} solution of DAB (pH 3.8) for 8 h under light at 25 °C. Leaves of DAB-treated plants were wounded 1 - 3 times perpendicular to the main vein by crushing with a hemostat. Immediately after wounding, the plant or leaves were continually supplied with DAB solution until the experiments were terminated by immersion of the plants or leaves in boiling ethanol (96 %) for 10 - 30 min. This treatment decolourized the leaves except for the deep brown to black polymerization product produced by the reaction of DAB with H_2O_2. After cooling, the leaves were extracted at room temperature in 96 % ethanol. For videodocumentation, leaves were first partially rehydrated in water, and then scanned using a flat-bed colour scanner (*ScanJet 3200 C, Hewlett-Packard*, Palo Alto, USA).

Alternativelly, a highly sensitive chemiluminescent detection of H_2O_2 for *in situ* purposes was performed as follows. Horse radish peroxidase (100 U cm^{-3}, *Sigma*, Deisenhofen, Germany) and *SuperSignal West Pico* solution (*Pierce*, Rockford, USA) were simultaneously infiltrated to the excised plants or leaves and incubated under light for a period of 2 h. A video imaging (VIM)-intensified cooled colour CCD camera (model *Progressive 3*, *Sony*, Tokyo, Japan) set to a longer integration times was used for chemiluminescence imaging. Analysis of images was performed using the *Adobe PhotoDeluxe* software (v. 2.0, *Adobe*, San José, USA).

Salicylic acid (SA, 1 mM) as well as both extracellular elicitor 1 and cell wall-derived elicitor 2 from a necrotrophic fungal pathogen *Botrytis cinerea* (Pers. et Fries) at concentration of 5.5 and 2.4 μg cm^{-3}, were supplied to the excised leaves in solution of DAB as described above and incubated under light until the experiments were completed. DAB-treated leaves were exposed to MeJA for the times indicated and then assayed for H_2O_2 generation.

Modulation of the ROS generation system and pharmacological experiments: Several compounds were screened for their potential activating or inhibiting activity on signal transduction and ROS generation after elicitation with MeJA: 100 μM NaN_3 (target, a wide range of redox enzymes) from stock solution dissolved in water; 1 mM DDC (target, SOD), and 1 - 4 mM SHAM (target, PRX) from stock solutions dissolved in ethanol; and 200 μM DPI (target, NADPH oxidases) from a stock solution in DMSO and purchased from *Sigma* (Deisenhofen, Germany). Where required, 100 U cm^{-3} of CAT (thymol free, *Sigma*) were vacuum infiltrated to the upper parts of leaves to remove H_2O_2, whereas 1 mM ATZ or 1 mM SA were added to inhibit endogenous CAT (Levine *et al.* 1994, Durner and Klessig 1996). All compounds at the concentrations indicated were supplied to excised plants or leaves previously incubated in DAB for 8 h. The plants or leaves were then locally inoculated with MeJA at a final concentration of 50 μM and continually supplied with the DAB solution for 2 - 8 h under light, when the plants/leaves were treated to visualize H_2O_2.

Statistical analysis: Data were analyzed by appropriate Student's *t*-test or other analyses of variance using *Microsoft Excel* (v. 5.0). Significant difference between individual treatments were determined using *LSD*.

Results

MeJA-induced hypersensitive-like cell death and H_2O_2 accumulation: Treatment of excised oak leaves with 50 μM MeJA caused a rapid cell death that mimic a typical hypersensitive response (HR) (Fig. 1). The tissue became slightly bright within 1 - 3 h, then became necrotic after 8 h. Histochemical localization of H_2O_2 in excised leaves allowed to imbibe solution of luminol/HRP or DAB (1 mg cm^{-3}) for 2 or 8 h, respectively, prior treatment with MeJA showed an accumulation of this reactive oxygen intermediates in cells surrounding the necrotic lesion (Fig. 1). A relative high amount of H_2O_2 was also found in other compartments of MeJA-treated leaves primarily in the vascular tissues (Fig. 1). However, mock inoculation with water did not produce substantial amounts of H_2O_2 in control leaves, suggesting that H_2O_2 accumulation in elicited leaves was produced in response to MeJA.

A survey of the MeJA induction of both HR and H_2O_2 in 12 species of plants from twelve plant families was conducted. Results obtained indicate that in response to MeJA treatment the excised leaves of all species tested exhibit visually observable H_2O_2 generation, assessed by DAB coloration (Fig. 2). MeJA-generated H_2O_2 was primarily localized in the veins and accumulated to a various extents. Surprisingly, MeJA-induced necrotizing response and HR-associated H_2O_2 generation were an exclusive characteristics of woody plant species (Fig. 2*A*). Six herbaceous species locally-treated with MeJA exhibited any HR-like symptoms and did not generate the HR-associated H_2O_2 (Fig. 2*B*).

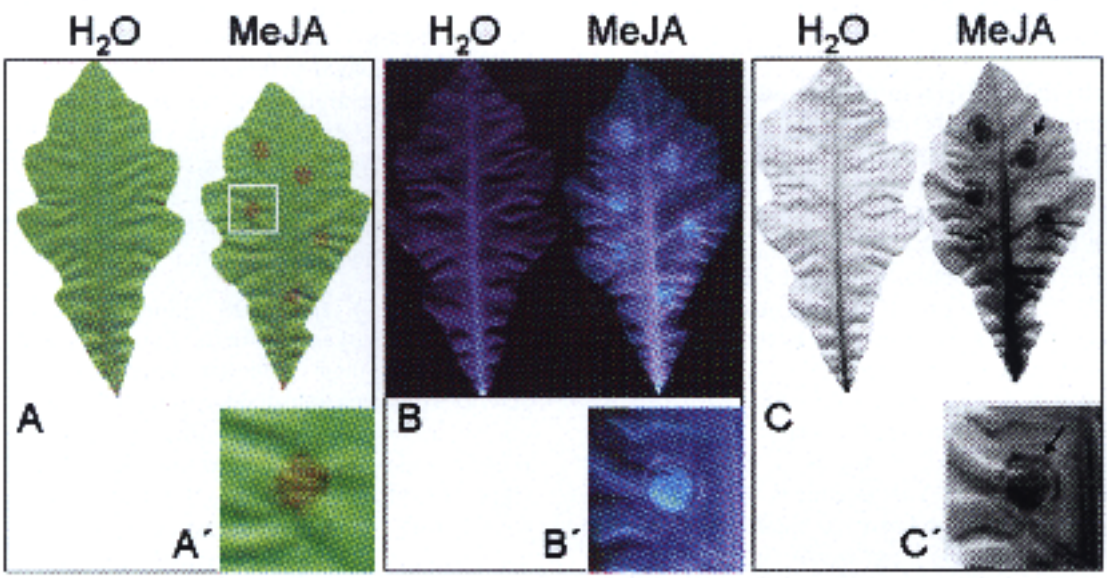

Fig. 1. The generation of H_2O_2 in leaves of oak plants in response to 50 μM MeJA. Leaves were excised at the base of the petioles and either vacuum infiltrated with the mixture of luminol/HRP for 2 h or supplied with DAB for 8 h. The leaves then were treated with droplets of MeJA. MeJA-induced HR-like lesion production 8 h after the onset of the treatment (*A*). The production of H_2O_2 in leaves of oak plants 8 h after treatment with MeJA and detected with chemiluminescence (*B*). The production of H_2O_2 in leaves of oak plants 8 h after treatment with MeJA and visualized histochemically with DAB (*C*). *Inserts* represent a higher magnification of the boxed area bearing necrosis depicted in 1*A*. *Arrows* in *C* and *C´*denote the localization of H_2O_2 in cells surrounding the HR-like lesions.

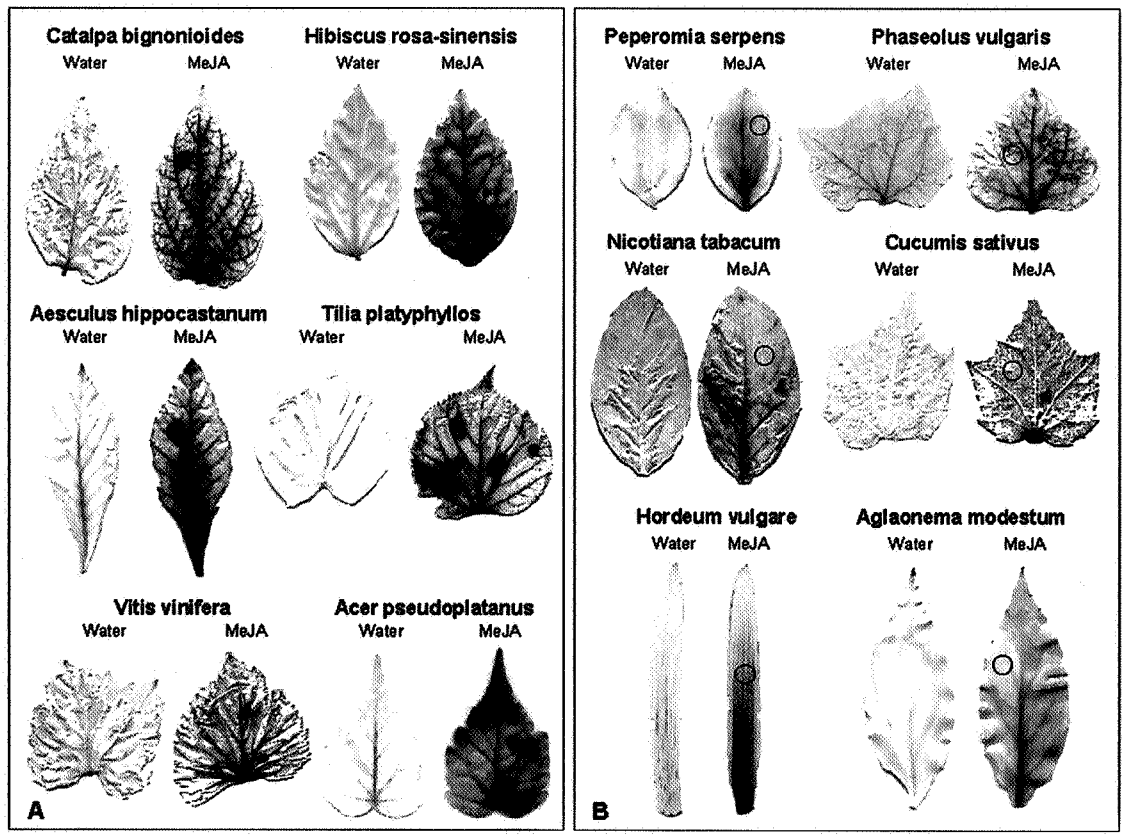

Fig. 2. Assay for MeJA-inducible H_2O_2 production in leaves of woody (*A*) and herbaceous (*B*) plant species from 12 plant families. Leaves of plants were excised and supplied with a solution of DAB for 8 h, then treated with 50 μM MeJA and assayed after an additional 8-h incubation with DAB. The *circles* in *B* indicate the boundaries of the MeJA-treated areas.

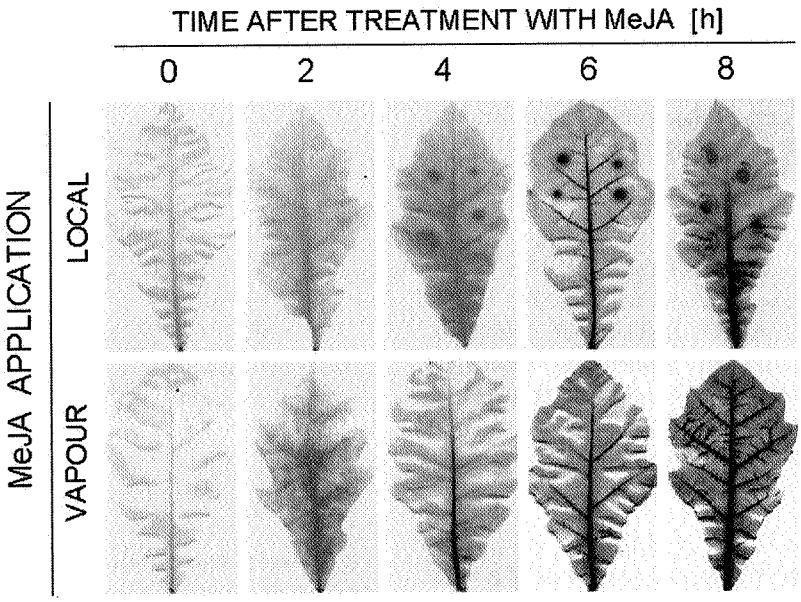

Fig. 3. The time course of H_2O_2 production in oak leaves in response to local or ectopic MeJA application. Excised leaves were first supplied with a solution of DAB for 8 h, then treated with 50 μM MeJA and assayed after an additional time points indicated for H_2O_2 generation.

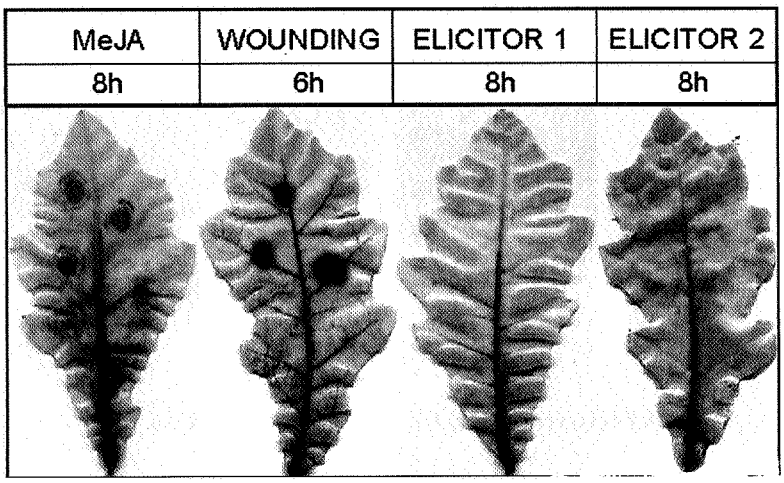

Fig. 4. The generation of H_2O_2 in leaves of oak plants in response to MeJA and other stress factors. The leaves were excised and supplied with a solution of DAB for 8 h. Then the leaves were either wounded, supplied with a solution of elicitor 1 (5.5 μg cm^{-3}) and elicitor 2 (2.4 μg cm^{-3}) in DAB through the cut petioles, or exposed to 50 μM MeJA at the times indicated and assayed.

Histochemical detection of H_2O_2 at interaction sites: MeJA-generated H_2O_2 production was kinetically analyzed in both locally- and ectopicaly-treated oak leaves. When MeJA was locally applied to the excised leaves H_2O_2 was detectable as early as 4 h after treatment, with the colour deepening for about 2 - 4 h (Fig. 3). The DAB-H_2O_2 reaction product was first visible at the site of MeJA application, then appeared also in major veins throughout the leaves. Within 8 h after the onset of the treatment, DAB detected H_2O_2 in cells surrounding the necrotic spots. Unlike to this, exposure of excised oak leaves to gaseous MeJA for 2 h was sufficient to induce a thousands of microbursts in leaf periveinal cells. With increasing time of exposure to the volatile MeJA the accumulation of H_2O_2 appeared in major veins (4 - 6 h) and approached a maximum at about 8 h when the DAB-H_2O_2 polymerization product was heavily detected in both major and minor veins (Fig. 3). Thus gaseous MeJA did not induce an HR-like symptoms.

Excised oak leaves were allowed to imbibe a 1 mg cm^{-3} solution of DAB for 8 h, then wounded or supplied with extracellular elicitor (Elicitor 1), or cell wall-derived elicitor (Elicitor 2) from a necrotrophic fungus *Botrytis cinerea*. H_2O_2 is detectable as early as 1 h after wounding (data not shown), with the colour deepening for about 6 h, then declining. The colour was visible in both at the

wound site and also appeared in major and minor veins (Fig. 4). Supplying oak leaves with both elicitors also induce the production of H_2O_2 primarily in major veins (Fig. 4). It is important to note that both elicitors induced a typical HR response but it appearance was highly delayed when compared to that induced by MeJA (data not shown).

MeJA-induced systemic accumulation of H_2O_2: Within 6 - 8 h after the primary treatment with 50 μM MeJA, H_2O_2 is shown to be present also in upper, untreated, leaves of young oak plants that were locally-treated on the lower leaf, indicating that the generation of H_2O_2 production was systemic. In these leaves the H_2O_2 appears to have spread throughout the major veins (Fig. 5).

Effect of inhibitors and scavengers on H_2O_2 production and the HR: The general inhibitor of a wide range of redox enzymes, NaN_3 (100 μM), did not have a significant effect on H_2O_2 production in control, water-treated, oak leaves (Fig. 6*A*). Preventing the oxidative burst via NaN_3 application completely abolished cell death induced by MeJA (Fig. 6*B*), suggesting that some of the ROS (H_2O_2 or O_2^-) is involved in this response.

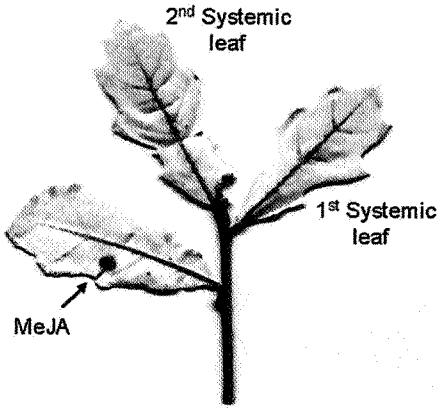

Fig. 5. The systemic production of H_2O_2 in excised oak plants treated with 50 μM MeJA only on the lower leaf. Thirty-d-old plants were excised at the base of the stem and supplied with a solution of DAB for 16 h. The plants then were treated with MeJA and continually supplied with DAB for an additional 8 h and assayed.

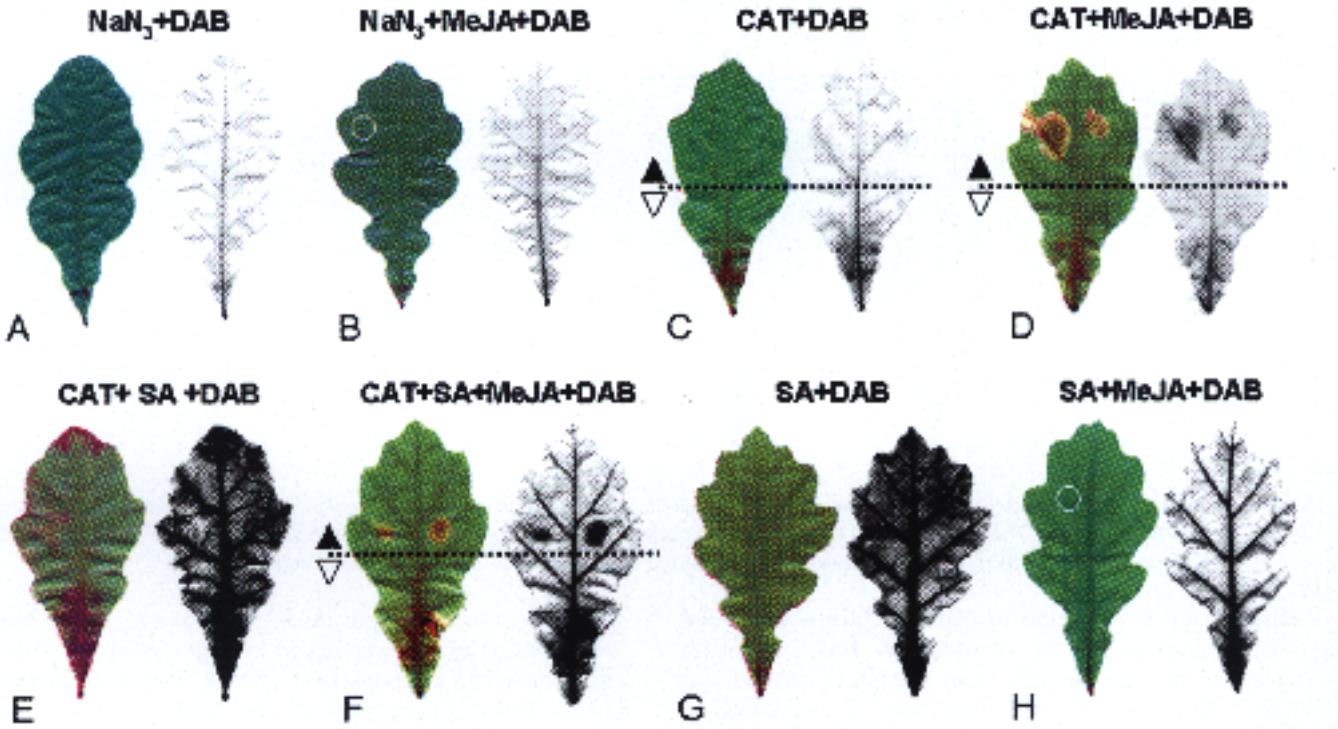

Fig. 6. Progression of damage and accumulation of H_2O_2 in inhibitor- and MeJA-treated oak leaves. Excised leaves were supplied with a solution of DAB containing the compounds indicated for 8 h. The leaves then were exposed to 50 μM MeJA as indicated followed by an additional incubation in a solution of DAB for 8 h and assayed. The horizontal dashed lines in the experiment *C*, *D*, and *F* demarcate the CAT infiltrated (*full triangle*) or CAT-untreated (*open triangle*) parts of the respective leaf. The circles in the experiment *B* and *H* indicate the boundaries of the MeJA-treated areas.

The infiltration of CAT (500 U cm^{-3}) into the upper part of the control leaves completely prevented accumulation of H_2O_2 whereas a substantially higher amounts of H_2O_2 were detected in the lower, unprotected, part of the respective leaves (Fig. 6*C*). The addition of MeJA to the CAT-protected part of leaves caused a

spread necrosis in the absence of H_2O_2 production (Fig. 6*D*), indicating that H_2O_2 is not necessary to this phenomenon.

Supplying SA (1 mM) to the excised oak leaves through their cut petioles strongly generated H_2O_2 in both vascular tissues and a numerous necrotic spots (Fig. 6*E*). This reflects the inhibition by SA of endogenous and exogenous CAT activity thus increasing the steady-state concentration of H_2O_2. The addition of MeJA to CAT+SA pre-treated part of leaves significantly lowered the amount of H_2O_2 when compared to the CAT-unprotected part of the same leaves. Moreover, although MeJA rendered its ability to induce the HR-like response, the shape of necroses was highly restricted (Fig. 6*F*). The 1 mM SA itself has no significant effect on the HR, instead expressed the inhibitory effect on endogenous CAT by increased accumulation of H_2O_2 (Fig. 6*G*). Pre-incubation of excised oak leaves in the presence of 1 mM SA completely abolished HR-like response induced by MeJA and reduced the amount of H_2O_2 produced (Fig. 6*H*).

Pharmacological analysis of the role of H_2O_2 in MeJA-induced HR-like response: Addition of an alternative CAT inhibitor, ATZ (1 mM), yielded in a substantial accumulation of H_2O_2 in both water- and MeJA-treated leaves whereas did not affect either the appearance of necrotic response or O_2^- content (Fig. 7). Almost the same results were obtained when SHAM (1 mM), an inhibitor of cell wall-bound PRXs, was supplied to the excised oak leaves (control or MeJA-treated, Fig. 7).

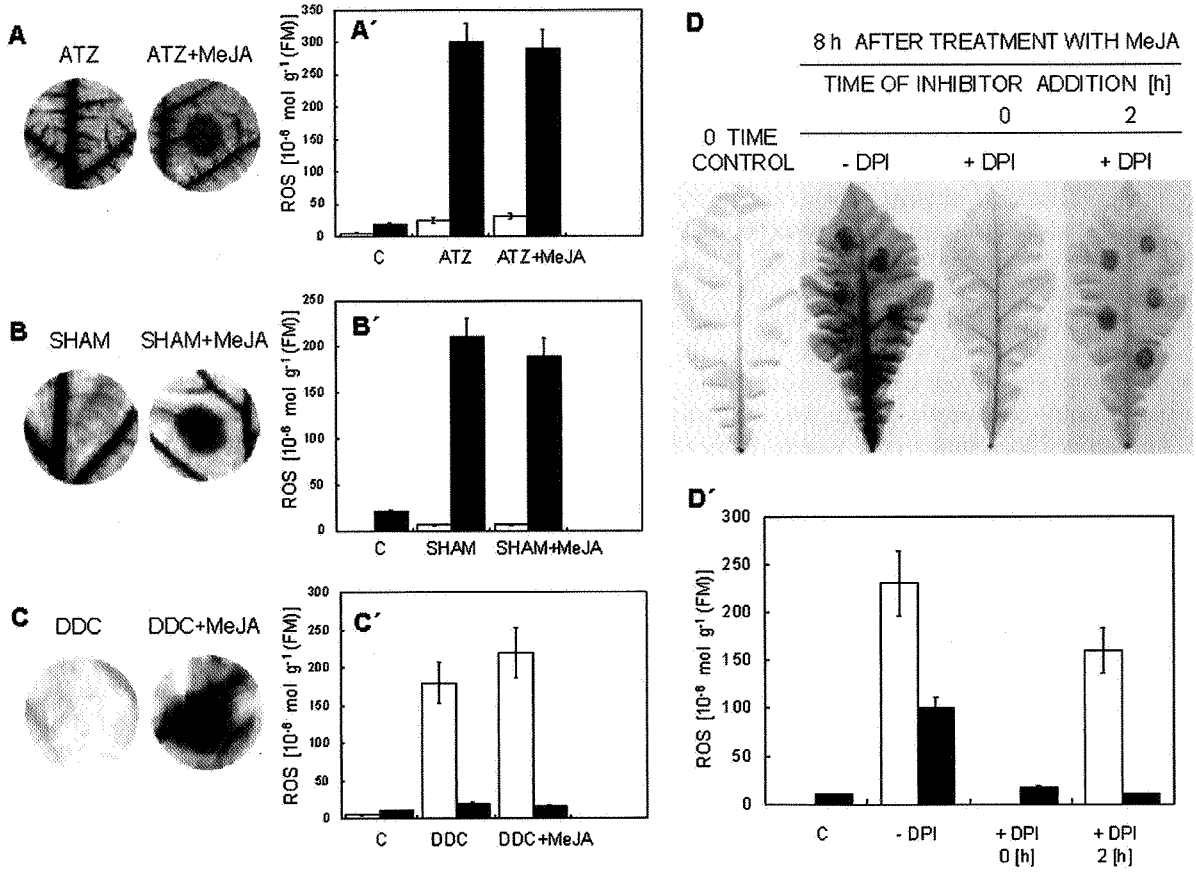

Fig. 7. The effect of possible source enzyme inhibitors on MeJA-induced HR-like cell death and ROS production. 0.1 cm^3 of 1 mM ATZ (*A*), 1 mM SHAM (*B*), or 1 mM DDC (*C*) was added to a DAB solution and supplied to the excised oak leaves for 8 h and assayed for MeJA-induced H_2O_2 production and HR response. Another part of leaves treated by the same manner were monitored either for O_2^- (*open column*) or for H_2O_2 (*full column*) production (*A′*, *B′*, *C′*). 200 μM DPI was supplied in a solution of DAB for 0 or 2 h after the treatment with MeJA, then continually supplied with DAB for an additional 8 h and assayed either for HR-like response (*D*) or ROS production (*D′*). O_2^- (*open column*) and H_2O_2 (*full column*) monitored over a period of 8 h. The measurements were repeated at least four times and *bars* indicate SE.

When the Cu/ZnSOD inhibitor DDC (1 mM) was supplied to water-treated leaves, there was no increase in H_2O_2 detection but a marked accumulation of O_2^- was observed. The addition of MeJA to DDC pre-treated leaves resulted in induction of both spread necrosis and O_2^- accumulation in the absence of its dismutation

product accumulation (Fig. 7).

DPI (200 μM), a "suicide" substrate inhibitor of mammalian NADPH oxidase, was supplied to DAB pre-treated leaves for 0 or 2 h after MeJA application. Simultaneous treatment of excised leaves with both DPI and MeJA resulted in no HR-like lesion formation (Fig. 7*D*) and no O_2^- accumulation (Fig. 7*D*), indicating that O_2^- rather than H_2O_2 might be responsible for MeJA-induced HR-like cell death. Additionally, no H_2O_2 was generated in this experiment, confirming that the oxidase causing the generation of H_2O_2 in response to MeJA exposure was inhibited. When the DPI was supplied to the leaves with a lag of 2 h after MeJA treatment the HR-like response similar to non-inhibited (-DPI) leaves appeared. Since DPI also blocked the production of H_2O_2 and the O_2^- content was still relatively high supporting the hypothesis that the latter may be a key element in MeJA-induced HR-like cell death.

Discussion

Exogenous MeJA applied directly onto the adaxial surface of excised oak leaves caused the formation of lesions that closely resemble hypersensitive response lesion that are characteristic of several incompatible plant-microbe interactions. The MeJA treatments induced an HR-like symptoms also in other woody plant species, but surprisingly did not induce any visible macroscopic changes in herbaceous plant species tested. These observation suggest the hypothesis that a highly sensitive and selective perception system for MeJA has evolved in woody plants. Thus, our ongoing work concentrates on the molecular characterization of this perception system and its role in the disease response signaling.

The oxidative burst has often been described as causing the HR cell death (Levine *et al.* 1994, Wojtaszek 1997, Desikan *et al.* 1998, Kazan *et al.* 1998). In our system, there was also no correlation between high H_2O_2 accumulation and cell death. Regardless of whether MeJA was applied as a liquid or a vapour, there was a common type of response to treatment. First, the early H_2O_2 burst was detected in the leaf periveinal cells histochemically observed as a plethora of microbursts and later H_2O_2 production appeared to be concentrated primarily in both vascular system and space surrounding necrotic lesions. Therefore, one may speculate that such a sequential pattern of H_2O_2 production caused by MeJA is a reminiscent of a biphasic oxidative burst triggered by the elicitin cryptogein (Pugin *et al.* 1997, Simon-Plas *et al.* 1997), treatment with a crude *Phytophthora soja* cell wall preparation (Jabs *et al.* 1997), and ozone-exposition of Bel W3 tobacco, which is known as an ozone biomonitor (Schraudner *et al.* 1998).

The histochemical dye DAB visually localized stress-inducible H_2O_2 not only in MeJA-treated oak leaves. Wounding and two distinct elicitors derived from a necrotrophic fungal pathogen *Botrytis cinerea* also induced the production of H_2O_2 in leaf veins similar to MeJA, indicating the similarities between the signaling for the production of H_2O_2 from the oxidative burst. Plants are capable of differentially activating distinct defense pathways, depending on the inducing agents. Jasmonate, SA, and ethylene play an important role in this signaling network. Cross-talk among these signaling pathways is thought to play a key role in fine-tunning complex defense responses (Reymond and Farmer 1998, Glazebrook 1999, Maleck and Dietrich 1999, Pieterse and Van Loon 1999).

H_2O_2 can act as a local signal for HR-cell death and also as a diffusible signal for the induction of defensive genes in adjacent cells (Alvarez *et al.* 1998, Bergey *et al.* 1999, Orozco-Cardenas and Ryan 1999). In this study, I have also shown that it is possible to trigger the systemic production of H_2O_2 by exogenous application of MeJA. The systemic character of the inducible H_2O_2 generation by MeJA bears strong resemblance to that described for response to wounding (Orozco-Cardenas and Ryan 1999). In the light of previously published results, the systemic production of H_2O_2 by MeJA is curious because MeJA alone appears not to be sufficient as an inducer of SAR (Cohen *et al.* 1993). This suggest the view that it may act in combination with other systemic signaling components such as SA and/or ethylene (Reymond and Farmer 1998, Dong 1998).

In an attempt to unravel a putative role of H_2O_2 in the HR-like response induced with MeJA treatment, the gain and loss of function experiments based on the blocking or the mimicking of the H_2O_2 burst were performed. NaN_3 at the concentration not toxic to the cells effectively blocked the induction by MeJA of the oxidative burst and HR-like response as well. This observation indicates that some of ROS are essential in the MeJA-induced cell death. CAT treatment completely block MeJA-induced H_2O_2 production but did not prevent MeJA-induced cell death suggesting that H_2O_2 from the oxidative burst is apparently not necessary in this process. Consequently, addition of 1 mM SA which can bind and inhibit the H_2O_2-removing enzyme CAT (Durner and Klessig 1996) highly restricted the MeJA ability to induce the both oxidative burst and HR-like necrotic response. SA has also been shown to increase SOD levels within 2 h of treatment *in planta* (Rao *et al.* 1997), and therefore a slight increase in H_2O_2 due to increased SOD activity might be expected. In fact, results in this paper confirmed such an expectation (Fig. 6*G,H*).

There have been conflicting results concerning the relationship between SA and MeJA effects. While in the former case addition of SA only reduced the shape of necrotic lesions induced with MeJA, in the latter one SA

completely abolished the necrosis-inducing effect of MeJA. There are two conclusions to be considered. First, due to binding SA to both endogenous and exogenously added CAT, there was not enough amount of free SA to block MeJA-induced ROS production completely. Second, SA was shown to block the biosynthesis of jasmonates via the inhibition of the conversion of 13-S-HPLA to 12-oxo-PDA (Farmer *et al.* 1994, Doares *et al.* 1995), where it likely inhibits the enzyme 13-S-hydroperoxide dehydrase (Pena-Cortés *et al.* 1993). Thus, SA compromised the octadecanoid defense signalling pathway, indicating that MeJA-induced HR-like cell death must require a functional octadecanoid pathway which can not be overcomed by exogenous jasmonate.

A hypothesis was proposed in which O_2^- rather than H_2O_2 would potentiate MeJA-induced HR-like cell death. In this context, the addition of an alternative CAT inhibitor ATZ or PRX inhibitor SHAM did not significantly affect the HR-like response induced with MeJA. In contrast, the Cu/ZnSOD inhibitor, DDC, dramatically reduced the generation of H_2O_2 whereas it does not compromise the HR-like cell death. Moreover, there was an apparent correlation between the extent of necrotic spots and the production of O_2^-, supporting the hypothesis that H_2O_2 from the oxidative burst is neither necessary nor sufficient in MeJA-induced HR cell death. Consistent with this statement are results with DPI, since DPI blocks NADPH oxidases, which are involved in production of O_2^- that is readily dismuted into a more stable H_2O_2. In general, the lack of O_2^- production through DPI activity may explains the inhibition of MeJA-induced HR-like cell death. Delayed addition of DPI was not sufficient to prevent the HR-like cell death induced with MeJA while the H_2O_2 generation was still almost completely blocked. From these observations, one may find a clue to the regulatory pathway involved in MeJA-induced HR-like response. Interestingly, other studies have also involved O_2^- rather than H_2O_2 as an essential component involved in defence response activation (Jabs *et al.* 1996, Jabs *et al.* 1997, Overmyer *et al.* 2000). However, such a putative role for O_2^- in the above discussed process remains to be further demonstrated.

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