J Plant Growth Regul (1988) 7:203–211

Journal of
Plant
Growth
Regulation

Jasmonic Acid–Dependent Increases in the Level of Specific Polypeptides in Soybean Suspension Cultures and Seedlings

James Michael Anderson

United States Department of Agriculture, Agricultural Research Service, Departments of Crop Science and Botany, North Carolina State University, Raleigh, North Carolina, 27695-7631, USA

Received June 24, 1987; accepted March 4, 1988

Abstract. Adding 2–30 μM jasmonic acid (JA) to photomixotrophic suspension cultures of soybean increased the level of several soluble polypeptides isolated by SDS-PAGE. The major polypeptides affected by JA treatment were at M_r 31,200 (p31) and M_r 39,000. Spraying leaves of soybean seedlings with 10–50 μM JA also increased the level of several soluble polypeptides including p31. The use of Con A affinity chromatography demonstrated that p31 was a glycoprotein and that JA increased the level of three other glycoproteins at M_r 22,000, 33,000, and 52,000. The JA treatment did not alter the growth or morphology of the seedlings. JA at 2–30 μM did not significantly inhibit the growth of the cultured cells and did not significantly alter the chlorophyll concentration. However, JA at concentrations above 30 μM inhibited growth and chlorophyll levels in cultured cells. The suspension cultured cells could provide a reliable bioassay for jasmonic acid.

Jasmonic acid and its methyl ester have been isolated from several families of plants, and the wide occurrence of JA and JA-Me suggests that these compounds may have a physiological role in plants (Anderson 1985, Meyer et al. 1984, Ueda and Kato 1980, Yamane et al. 1981). Support for a hormonal role

This paper represents cooperative investigations of the U.S. Department of Agriculture, Agricultural Research Service, and the North Carolina Agricultural Research Service, Raleigh, North Carolina 27695-7601. Paper No. 10997 of the journal series of the North Carolina Agricultural Research Service, Raleigh, NC 27695-7601.

for JA is provided by the similarities between JA and the prostanoids in both chemical structure and method of synthesis (Vick and Zimmerman 1987, Needleman et al. 1986). The prostanoids are an important class of stress-related hormones in animals (Wolff 1982). A plant growth regulatory role for JA is also supported by chemical and biological similarities to ABA (Ueda and Kato 1981). Both ABA and JA are keto acids with similar molecular weights, solubility properties, and pK's. Also, both compounds inhibit plant growth and promote the senescence of detached leaves (Dathe et al. 1981, Miersch et al. 1986, Ueda et al. 1981, Udea and Kato 1982, Yamane et al. 1981). Although JA has been shown to be more effective than ABA in promoting the senescence of detached leaves, a specific physiological role for JA has yet to be defined.

Suspension cultures have many advantages for biochemical investigations including fairly uniform cell populations and reproducible growth. However, most cell cultures do not give a clearly defined physiological response to added plant growth regulators. We recently found that treatment of the cultured soybean cells with JA increases the level of several polypeptides. This report characterizes this JA-dependent response in photomixotrophic suspension cultures.

Materials and Methods

Plant Material

Seeds of *Glycine canescens* Hermann PI 440.930 were obtained from Dr. R. L. Bernard, Department of Agronomy, University of Illinois. Seedlings of (*Glycine max* Merr. var. Williams 79) were grown in a greenhouse with a 15-h day length. Sunlight was supplemented with 1000-W metal halide lights (General Electric Co.) at the end of the photoperiod. When the unifoliate leaf was fully expanded, plants were sprayed at the end of the photoperiod for 5 days with 10–50 μM JA in Tween 80 0.05% v/v. Controls of Tween 80–treated and untreated were included. After 120 h of treatment, leaves were harvested, frozen in liquid N_2, and lyophilized.

Tissue Culture

Suspension cultures of soybean (*G. max* var. Corsoy) were supplied by Dr. Jack Widholm, University of Illinois. Cultures were grown photomixotrophically on PRB medium (Horn et al. 1983), which was modified by adding sucrose at 11 mM and substituting Zn EDTA for $ZnSO_4$. Culture conditions were as follows: 28°C, 3% CO_2 enriched air, cool white fluorescent illumination (fluence rate 350 μE m^{-2}), subculture on 7-d cycle, and rotation at 250 rpm. Heterotrophic cultures were grown in 84 mM sucrose in the absence of light and CO_2. Filter-sterilized JA was added at 24 or 48 h after subculture, and experiments were terminated by collecting cells on nylon nets, rinsing with H_2O, and blotting dry. Tissue was frozen in liquid N_2 and lyophilized. The

growth of each suspension culture was routinely measured by allowing cultures to settle for 20 min in 100-ml graduates. Data are expressed as the change in cell volume.

Protein Extraction

Lyophilized plant material (0.1 g) was repeatedly extracted with ice-cold acetone using a ground-glass homogenizer until free of visible chlorophyll. The resulting powder was dried on filter paper and homogenized in 5 ml of ice-cold 50 mM TRIS-HCl (pH 7.6), 3 mM EDTA, 1 mM PMSF, and 700 μM β-mercaptoethanol. Homogenates were filtered through four layers of cheesecloth, and any precipitate was removed by centrifugation at 18,000*g* for 20 min. A glycoprotein fraction was isolated from soluble extracts using a modification of the methods of Wittenbach (1983). The steps used included $(NH_4)_2SO_4$ precipitation with retention of the fraction that precipitated between 1.7 and 3.3 M $(NH_4)_2SO_4$, and Con A–Sepharose affinity chromatography using 100 mM acetate buffer, pH 5.9, containing 0.15 M NaCl. The loaded column was washed with running buffer until the A_{280} was less than 0.008 and the glycoprotein fraction was eluted using 50 mM 1-*O*-methyl-α-D-glucopyranoside in running buffer.

Polyacrylamide Gel Electrophoresis

SDS-PAGE was done by the methods of Laemmli (1970). Samples of 90 μg as determined by the method of Bradford (1976), were separated in 13% acrylamide by electrophoresis. Protein bands were resolved with Coomassie blue R-250 (BioRad) and individual lanes were cut from the gels and scanned at 560 nm. The area under the resultant peaks was determined by planimetry. Peak areas were normalized against a series of bands that did not change between treatments.

The experiments shown in Figs. 1–3 were repeated at least twice, and the data shown are from a single experiment that was representative of all similar experiments. In Fig. 4, data from separate experiments were not averaged, because the size of the response to JA was not consistent. However, the biphasic response of polypeptides to JA treatment shown in Fig. 4 was reproducible in three separate experiments.

Chemicals

Methyl jasmonate, (±)-JA-Me, was the kind gift of Dr. Gunther Ohloff of Firmenich SA, Geneva, Switzerland. (±)-Jasmonic acid, JA, was made by alkaline hydrolysis of (±)-JA-ME (1).

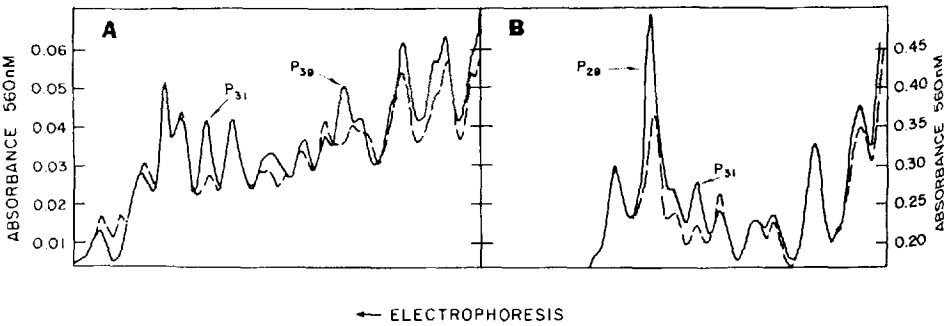

Fig. 1. Densitometric scans of parts of four separate SDS electophoretograms of soluble proteins extracted from tissues treated with JA (———) or untreated (– – –). Soybean cells grown photomixotrophically (panel A) were treated with 10 μM JA for 3 days. Fully expanded leaves of soybean seedlings (panel B) were sprayed with 50 μM JA for 5 days. Only the region of each between approximately M_r 45,000 and 25,000 is shown.

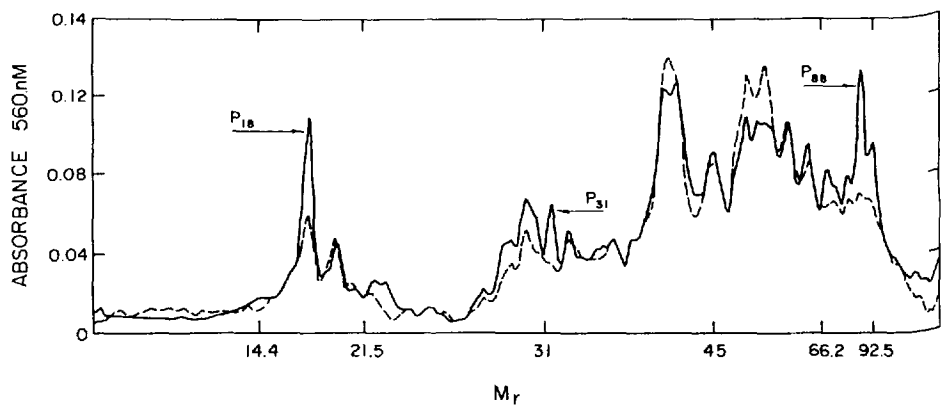

Fig. 2. Densitometric scan of SDS electrophoretogram of soluble proteins extracted from soybean cells grown heterotrophically in the dark. Jasmonic acid, 10 μM, was added after 3 days, and proteins were isolated after 48 h of JA treatment (———) or no treatment (– – –).

Results

Changes in Polypeptides

Jasmonic acid added at 1–30 μM increased the level of several polypeptides in soluble extracts of soybean cells grown photomixotrophically (Fig. 1A). The increase in polypeptides was easily quantified by densitometric measurements of the bands in an SDS-PAGE gel. The most obvious increase was in two polypeptides, at M_r 31,200 (p31) and at M_r 39,000 (p39). In contrast to JA, additions of 10 μM ABA, GA, or BA did not change the polypeptide pattern (data not shown). The JA-dependent increase in p31 was observed in over 15 experiments and occurred whether the JA was added to cells in log phase or stationary phase.

Spraying soybean seedlings with 10–50 μM JA for 5 days also increased the level of p31 in soluble extracts of fully expanded leaves (Fig. 1B) and another

polypeptide at M_r 29,500 (p29). Bands at both p31 and p29 were present in extracts from untreated leaves but at levels lower than treated leaves. The JA-dependent increase in p31 and p29 was also found to occur in *G. canescens* (data not shown). Jasmonic acid at 10–50 μM did not reduce the growth rate of any of the plants, and visual inspection of treated plants revealed no obvious changes in the plants relative to spreader-treated controls.

Since protease levels might be increased by JA treatment, leaf tissue and cultured cells were homogenized in the presence of PMSF. In addition, crude extracts without PMSF were incubated at 25°C for 2 h. In both experiments, the JA-dependent increase in p31 was still observed (data not shown).

Addition of JA to soybean cultures grown heterotrophically in the dark also increased the level of p31. Two additional bands at M_r 18,000 (p18) and M_r 88,000 (p88) were also increased (Fig. 2). Occasionally, p88 was slightly increased by JA in photomixotrophic cells, but the JA-dependent increase in these two bands was more evident in heterotrophic cells.

The p31 had an apparent molecular weight that varied between 29,000 and 31,000 depending on the buffer conditions used during electrophoresis. Therefore, the possibility that the p31 might be a glycoprotein was investigated. When PAGE gels were stained with a periodic acid–Schiff reagent (Matthieu and Quarles 1973), a band corresponding to the p31 appeared (data not shown). Also, p31 was retained by a Con A affinity column and required glucopyranoside for elution (Fig. 3A). In addition to p31, several other apparent glycoproteins were increased by JA treatment, and some of these changes were not apparent prior to isolation by Con A. The most significant changes in glycoprotein levels were at M_r 22,000 (p22), M_r 33,000 (p33), and approximately M_r 52,000 (p52). The increase in the level of p22 and p33 compared to p31 after the affinity purification is not known but may indicate that a portion of the p31 is lost on washing the column, because no p31 is left in the soluble fraction after passing through Con A (data not shown).

The increase in p31 in soybean cultures became apparent 24 h after addition of JA to the cultures and the level of p31 continued to increase until 48 h after JA addition. The increase in the level of p31 was directly related to the log of the JA concentration up to 30 μM JA (Fig. 4). Above 30 μM JA, the level of p31 was inhibited, but the level of p18 was increased with JA above 10 μM. This biphasic response to JA concentration was also apparent in the growth of the cultures. In no experiment was the growth rate changed by JA concentrations below 30 μM JA. However, growth inhibition was observed in at least three experiments at concentrations above 30 μM (data not shown). In fact, above 100 μM JA, growth was often completely inhibited, and the cultures turned brown.

Changes in Chlorophyll

Photomixotrophic cultures became a darker green color with JA treatment (1–10 μM), and isolated cells no longer adhered to glass surfaces. Visual observation with a light microscope of treated and untreated cells revealed no obvious morphological differences. Jasmonic acid at 1.0–10 μM sometimes

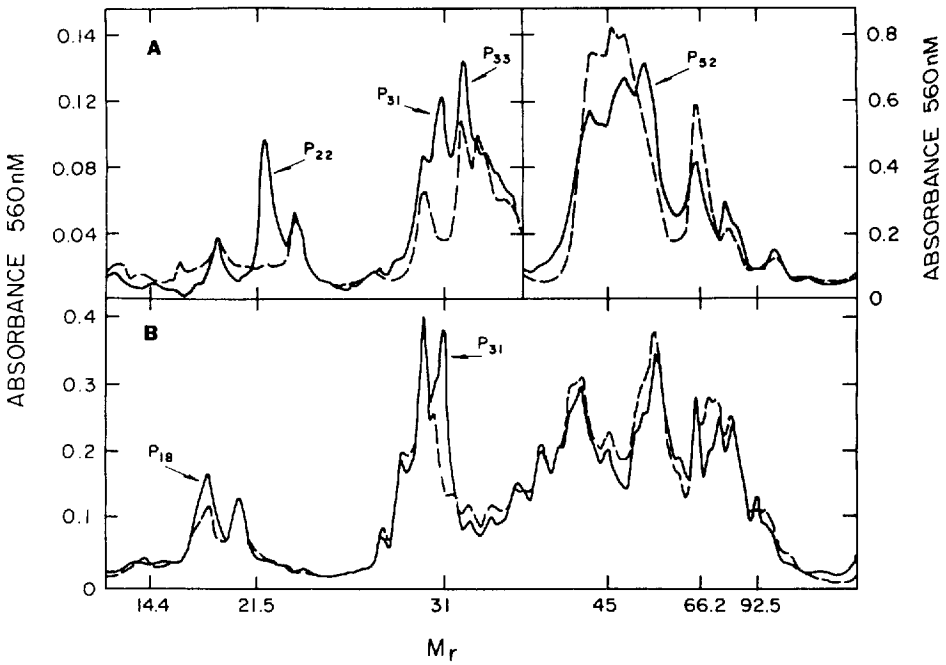

Fig. 3. Densitometric scan of SDS electrophoretogram of proteins extracted from soybean cells grown photomixotrophically. (A) 135 μg soluble proteins retained by Con A–Sepharose per well, and (B) 90 μg of soluble protein extract per well. Jasmonic acid, 10 μM, was added after 24 h, and proteins were isolated after 48 h of JA treatment (——) or no treatment (------).

caused a slight increase in the level of extractable chlorophyll, but at concentrations above 30 μM, JA inhibited chlorophyll synthesis (Fig. 5). This biphasic response of the cells to JA was also reflected in the JA-dependent changes in the chlorophyll A/B ratio (Fig. 5). The A/B ratio was not changed significantly until 30 μM JA. Above 30 μM, JA lowered the A/B ratio. Therefore, changes in the level of polypeptides, chlorophyll, and the rate of growth all exhibited a biphasic response to increasing levels of JA.

Discussion

The photosynthetic soybean suspension cultures are insensitive to the addition of any of the classical plant growth regulators, except auxin, which is required for growth. However, micromolar concentrations of JA added to the culture medium caused a major change in the level of several polypeptides. These changes were easily visualized in the stained electrophoretic gel. One of the proteins, p31, is a minor polypeptide in untreated cells but becomes one of the major soluble polypeptides in JA-treated cells. JA also increased the level of polypeptides in intact soybean leaves, which indicates that the JA stimulation may be a natural process. The change in p31 was less than in the cultures, but soybean leaves contain measurable levels of JA (unpublished results) and so may already be partly stimulated.

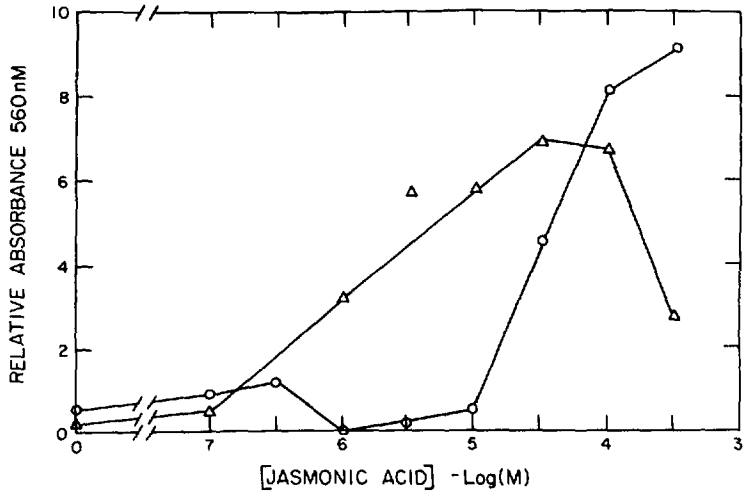

Fig. 4. Concentration dependence of the JA-dependent increase in polypeptides at M_r 31,200 (△——△) and at M_r 18,000 (○——○). Cells were treated for 48 h prior to isolating soluble proteins using SDS-PAGE. The p31 and p18 were measured relative to other protein bands in a SDS-PAGE gel using densitometry. Data are from a single representative experiment.

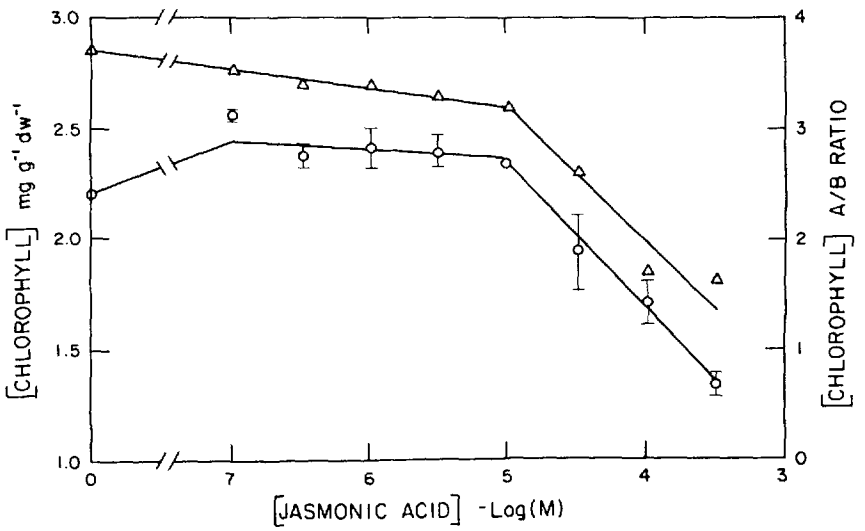

Fig. 5. JA-dependent changes in chlorophyll concentration (○——○) and chlorophyll A to B ratio (△——△). Cells were treated as described in Fig. 4. The data are an average of three separate experiments, with the SD shown by the error bars.

Most of the polypeptides increased by JA appear to be glycoproteins, because they bind to Con A and can be stained for carbohydrate. In fact, changes in the level of several glycoproteins in cultured cells were not apparent until after affinity chromatography. The identities of p29, p31, p52, and p88 have not been determined, but a group of similar glycoproteins (M_r 27,000, 28,000,

50,000, and 80,000) have been isolated from the paraveinal mesophyll (PVM) of soybean leaves (Franceschi et al. 1983, Wittenbach 1983). The slight difference in apparent molecular weights between the PVM proteins and JA proteins can be accounted for by the molecular weights assigned to the standards by Wittenbach (1983). The PVM glycoproteins are apparently leaf storage proteins that store nitrogen until utilization by developing seeds. The possible identity of the JA-increased proteins and PVM proteins is under investigation.

Several of the physiological effects that have been attributed to JA can also be elicited by ABA, often at lower concentrations than with JA (Tsurumi and Asahi 1985, Ueda and Kato 1981). This raises the possibility that JA increases specific polypeptides by inducing ABA synthesis. However, ABA added to soybean suspension cultures did not change the polypeptide profiles. It might also be noted that neither GA nor cytokinins had any effect on the polypeptide profile in either the presence or absence of JA.

The physiological function of jasmonic acid in plants has remained obscure in spite of several investigations over the last few years. Part of the problem has been that some of the bioassays for jasmonic acid require inordinately high concentrations of JA for a physiological response. This is particularly true of growth inhibition studies of dwarf rice and wheat seedlings, where 50 and 500 μM jasmonic acid, respectively, is required to obtain one-half maximal inhibition of growth (Dathe et al. 1981, Meyer et al. 1984). In contrast with growth inhibition studies, the stimulation of p31 in soybean suspension cultures requires only 2 μM JA to obtain one-half maximal stimulation (Fig. 5). Commercially available JA is a mixture of active and inactive isomers, so the actual concentration of active JA in the medium was probably less than 1 μM (Vick and Zimmerman 1987). The suspension cultures may therefore provide the first reliable bioassay for JA activity.

Neither growth inhibition nor loss in chlorophyll was observed at concentrations of JA that increased the level of p31 in either cultured cells or intact plants. However, growth inhibition was observed in cultured cells at a JA concentration greater than 10-fold the level required for p31 stimulation. Both JA and methyl jasmonate have been shown to possess senescence-promoting properties in leaf segments of oat and barley (Miersch et al. 1986, Satler and Thimann 1981, Ueda and Kato 1980). In these studies senescence was defined as a loss in chlorophyll and carotenoids. Again, JA did not cause a loss in chlorophyll in suspension cultures until the concentration was 10-fold higher than that required to increase p31. Therefore, the growth-inhibiting and senescence-promoting activities of JA may be related to toxic concentrations of JA rather than physiologically significant levels. Additional evidence against a role for JA in promoting senescence is the observation of Vick and Zimmerman (1986) that JA-biosynthetic enzymes appear to be found at higher levels in young, actively growing tissues rather than older tissues.

Acknowledgments. The excellent technical assistance of S. Dawson is appreciated.

References

Anderson JM (1985) Simultaneous determination of abscisic acid and jasmonic acid in plant extracts using high-performance liquid chromatography. J Chromatogr 330:347–355

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Dathe W, Ronsch H, Preiss A, Schade W, Sembdner G, Schreiber K (1981) Endogenous plant hormones of the broad bean, *Vicia faba* L. (–)–jasmonic acid, a plant growth inhibitor in the pericarp. Planta 153:530–535

Franceschi VR, Wittenbach VA, Giaquinta RT (1983) Paraveinal mesophyll of soybean leaves in relation to assimilate transfer and compartmentation. III. Immunohistochemical localization of specific glycoproteins in the vacuole after depodding. Plant Physiol 72:586–589

Horn ME, Sherrard JH, Widholm JM (1983) Photoautotrophic growth of soybean cells in suspension culture. I. Establishment of photoautotrophic cultures. Plant Physiol 72:426–429

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Matthieu J-M, Quarles RH (1973) Quantitative scanning of glycoproteins on polyacrylamide gels stained with periodic acid–Schiff reagent (PAS). Anal Biochem 55:313–316

Meyer A, Miersch O, Buttner C, Dathe W, Sembdner G (1984) Occurrence of the plant growth regulator jasmonic acid in plants. J Plant Growth Regul 3:1–8

Miersch O, Meyer A, Vorkefeld S, Sembdner G (1986) Occurrence of (+)-7-iso-jasmonic acid in *Vicia faba* L. and its biological activity. J Plant Growth Regul 5:91–100

Needleman P, Turk J, Jakschik BA, Morrison AR, Lefkowith JB (1986) Arachidonic acid biochemistry. Annu Rev Biochem 55:60–102

Satler SO, Thimann KV (1981) Le jasmonate de methyle: Nouveauet puissant promotteur de la sensescence de feuilles. C R Acad Sci Paris 293:735–740

Tsurumi S, Asahi Y (1985) Identification of jasmonic acid in *Mimosa pudica* and its inhibitory effect in auxin- and light-induced opening of the pulvinules. Physiol Plant 64:207–211

Ueda J, Kato J (1980) Isolation and identification of a senescence-promoting substance from wormwood (*Artemisa absinthium* L.). Plant Physiol 66:246–249

Ueda J, Kato J (1981) Promotive effect of methyl jasmonate on leaf senescence in the light. Z Pflanzenphysiol 103:357–359

Ueda J, Kato J (1982) Inhibition of cytokinin-induced plant growth by jasmonic acid and its methyl ester. Physiol Plant 54:249–252

Ueda J, Kato J, Yamane H, Takahashi N (1981) Inhibitory effect of methyl jasmonate and its related compounds on kinetin-induced retardation of oat leaf senescence. Physiol Plant 52:305–309

Vick BA, Zimmerman DC (1986) Characterization of 12-oxo-phytodienoic acid reductase in corn. The jasmonic acid pathway. Plant Physiol 80:202–205

Vick BA, Zimmerman DC (1987) Oxidative systems for modification of fatty acids: The lipoxygenase pathway. In: Stumpf PK, Conn EK (eds) The Biochemistry of Plants, Vol 9; Lipids. Academic Press, New York, pp 53–90

Wittenbach VA (1983) Purification and characterization of a soybean leaf storage glycoprotein. Plant Physiol 73:125–129

Wolff LS (1982) Eicosanoids: Prostaglandins, thromboxanes, leukotrienes, and other derivatives of carbon-20 unsaturated fatty acids. J Neurochem 38:1–14

Yamane H, Takagi H, Abe H, Yokota T, Takahashi N (1981) Identification of jasmonic acid in three species of higher plants and its biological activities. Plant Cell Physiol 22:689–697